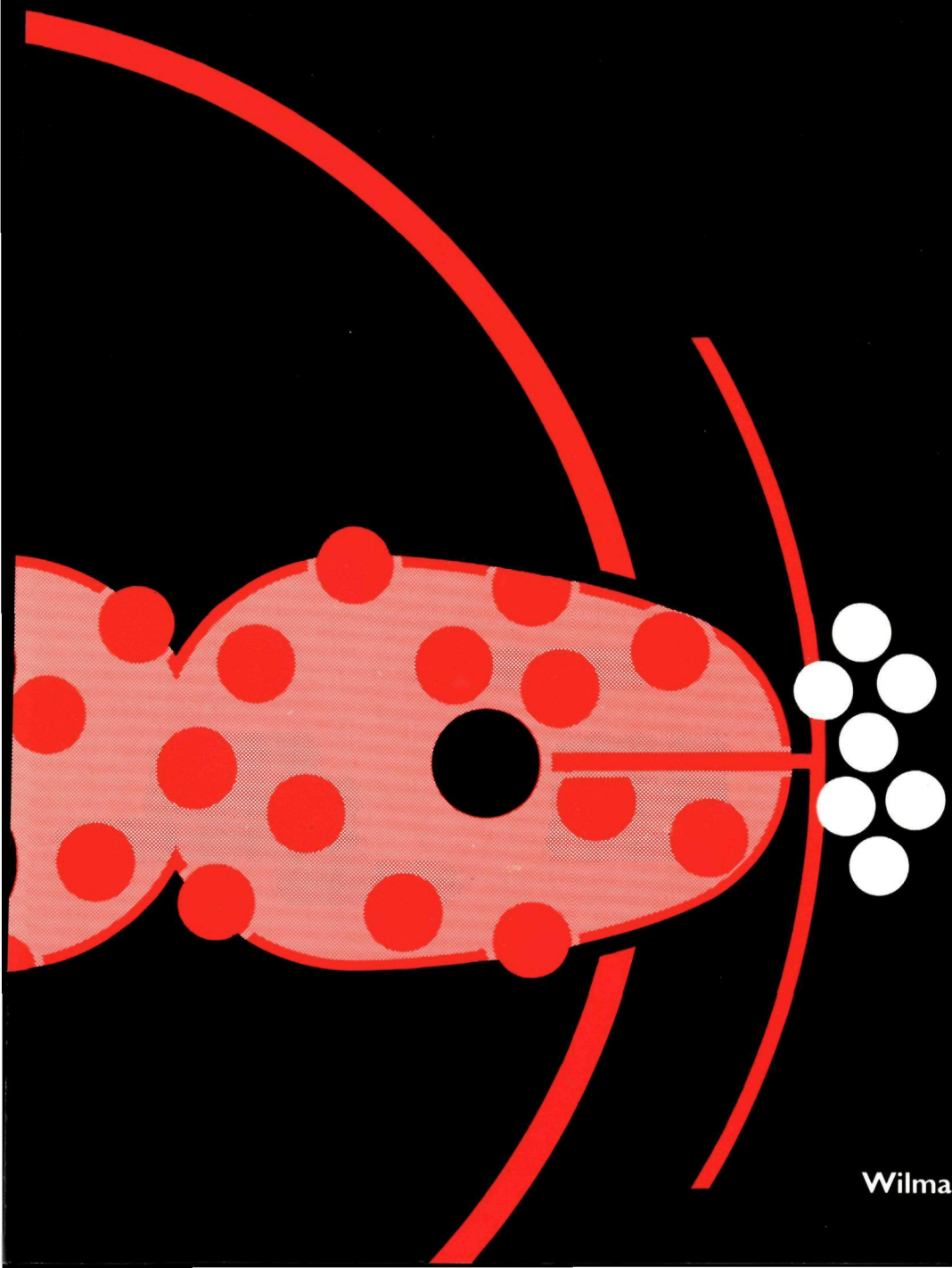
HATCHING IN DECAPOD CEPHALOPODS

STRUCTURAL, BIOCHEMICAL AND ENVIRONMENTAL ASPECTS



Wilma Paulij

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HATCHING IN DECAPODE CEPHALOPODEN

Structurele, biochemische en oecologische aspecten

omslag: Hanske van Westreenen

HATCHING IN DECAPOD CEPHALOPODS

Structural, biochemical and environmental aspects

een wetenschappelijke proeve op
het gebied van de natuurwetenschappen,
in het bijzonder de biologie

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PART I: Structural and biochemical aspects

PART II: Environmental aspects

GENERAL INTRODUCTION

Cephalopods form a class within the Mollusca, and with the exception of those belonging to the genus *Nautilus*, all living species are included in the subclass Coleoidea (Boyle, 1983; Roper et al., 1984). The main living coleoid orders are constructed along basically similar lines, but have certain characteristic morphological features and "life-styles" (Voss, 1977). The total number of living species of cephalopods is less than 1000 distributed over 43 families (Roper et al., 1984). Cephalopods occur in all marine habitats of the world and the range of depths extends from the surface to over 5000 m.

Squids and cuttlefishes

The common cuttlefish, *Sepia officinalis* Linnaeus 1758, and the European squid, *Loligo vulgaris* Lamarck 1799, are the most common cephalopods along the North Eastern Atlantic coasts (Grimpe, 1922; 1926; Kaas and Ten Broek, 1942; Von Boletzky, 1983; Worms, 1983). The veined squid *Loligo forbesi* Steenstrup 1856, is regularly found in British waters (Holme, 1974; Roper et al., 1984). These two groups of cephalopods, *i.e.* squids (order Teuthoidea) and cuttlefishes (order Sepioidea), are easily distinguished by external characteristics of the adults. The squids have an elongate, torpedo-like body with posterolateral fins, eight circumoral arms, not connected at the bases with a web, and two rows of stalked suckers. Two longer tentacles with an organized cluster (tentacular club) possess two or more rows of suckers at the distal end (Roper et al., 1984; Worms, 1983). Cuttlefishes have broad sac-like bodies with lateral fins that are either short, round and flap-like (Sepiolidae) or narrow and equaling the length of the mantle (Sepiidae) like in *S.officinalis*. The posterior lobes of the fins are free (subterminal) and separated by the posterior end of the mantle. In total ten arms are found. The longest (4th) pair, which form the actual tentacles, can retract into pockets at the ventrolateral sides of the head. The eyes are covered with a transparent membrane and eyelids are always present. In the cuttlefish the shell is composed of calcareous and horny matter (Roper et al., 1984; Tompsett, 1939).

Migration

Like many other cephalopods, *S.officinalis*, *L.vulgaris* and *L.forbesi* are migratory animals. In *S.officinalis* seasonal (mainly vertical) in-offshore migrations have been shown to occur in all stocks (Mangold, 1987; Roper et al , 1984). There are various forms of vertical migrations in pelagic species like *Sepia*, whereas many squids perform long distance migrations (Von Boletzky, 1989a). The *L.forbesi* population in the North Eastern Atlantic is known to carry out seasonal migrations, spending the summer in the North Sea and the eastern part of the English Channel and overwintering in the western part of the Channel (Holme, 1974). *L vulgaris* overwinters in deeper waters off Portugal, approaches the French coast in spring and migrates from May through June further north into the North Sea. A southward migration takes place in fall (Roper et al., 1984).

Reproduction

The migratory activity of cephalopods is mainly related to their reproduction but there is a great variability of spawning in terms of time of the year, size and age of reproducing cephalopods. Spawning ranges from strictly seasonal to year-round. Size and age at spawning may differ greatly within a species (Mangold, 1987). In the English Channel *L.forbesi* appears to be a strict seasonal spawner, with the bulk of the population reproducing in December-January in coastal waters (Holme, 1974). The age at spawning is one year. Observations of Von Boletzky and Mangold (1985) on a population in the North Western Mediterranean suggest that a similar reproductive rhythm

may occur in the milder conditions of this sea. In the French and Dutch coastal waters *S.officinalis* and *L.vulgaris* spawns are found from early spring to late summer (Mangold, 1987; Richard, 1971; Von Boletzky, 1983; Worms, 1983). The large *Sepia* females are the first to arrive on the breeding grounds and also the first to spawn. These females are about 18 months old (Von Boletzky, 1983) About 14-16 months old, smaller females arrive and spawn later (Mangold, 1987). The spawning period of *L.vulgaris* in the North Sea is five months (April-August) with a maximum during May (Tinbergen and Verwey, 1945). The female life span is 18 to 24 months, but males can live as long as 3 to 3.5 years (Worms, 1983).

Maturation

Cephalopods are gonochoristic, *i.e* the sexes are separated. In decapods the gonad appears as a single cell mass around the middle of embryonic development (Lemaire, 1972; Fioroni and Sundermann, 1983). Differentiation into testis or ovary appears not to be under hormonal control (Lemaire and Richard, 1979, Richard and Lemaire, 1975). Gametogenesis, especially oogenesis, has been investigated by many authors (for reviews see Arnold and Williams-Arnold, 1977; Wells and Wells, 1977a) but the most detailed study of male gamete development is probably by Richard (1971) for *S.officinalis*. The presence of free ova in the ovary indicates that the females are mature, while the presence of fully differentiated spermatozoa in the testis and the proximal vas deferens does not indicate that males are mature (Arnold and Williams-Arnold, 1977). The male is not ready to copulate until the spermatozoa have been packed into spermatophores (Mangold, 1987).

A hormone produced by the optic glands is probably one of the most important factors controlling maturation (Wells and Wells, 1959; 1977b). The presence of this hormone is necessary for vitellogenesis to occur (O'Dor and Wells, 1973) while in males it is necessary for the division of spermatogonia (Richard, 1971). A large number of studies indicate that maturation is also controlled by several environmental factors such as light (Laubier-Bonichon and Mangold, 1975; O'Dor et al., 1977, Richard, 1967; 1968; 1970; Wells and Wells, 1959; 1969; 1972), temperature (Richard, 1966a; 1966b) and nutrition or food availability (Mangold and Boucher-Rodoni, 1973, Rowe and Mangold, 1975; Von Boletzky, 1975; 1979b; Van Heukelem, 1976) Mangold and Froesch (1977), on the contrary, suggest that gonads in mediterranean octopods develop relatively independently of external factors.

Courtship

In most cephalopod species, but in particular in *Sepia*, courtship regularly occurs and is rather spectacular (Bott, 1938; Grimpe, 1926; Richard, 1971; Schroder, 1966, Tinbergen 1939; Von Boletzky, 1983; Zahn, 1975). Courtship, involving special colour patterns, and movements of the arms and the body has also been described for several loliginid species (Arnold, 1962; Drew, 1911; Fields, 1965; Hanlon, 1978; Hurley, 1977). In both *Sepia* and *Loligo*, the male is the more active sex and selects a female by exhibiting special colour patterns. *Sepia* copulates in the head-to-head position (Aristoteles position) but in loliginids two different modes occur During migration into shallow-water spawning sites the animals predominantly mate in the head-to-head position, the spermatophores being deposited on the buccal membrane where the sperm is stored in a pouch. Once the animals have reached the spawning grounds, the spermatophores are directly transferred into the mantle cavity. To achieve this, the male approaches the female from below and grasps her around the mantle. The hectocotylus then picks up spermatophores and introduces them into the mantle cavity (Drew, 1911; Fields, 1965; Mangold, 1987).

Spawning

In both *Sepia* and *Loligo* spawning shortly follows mating (Mangold, 1987; Von Boletzky, 1983). Coleoid cephalopods apparently reproduce only once and then die. To ensure optimal embryonic development, cephalopods protect their gametes by specific structures. Males produce spermatophores to enclose the spermatozoa while females form protective envelopes for the

eggs at the moment of spawning. Only in incirrate octopods the eggs have no envelopes, but they are protected by the females themselves (Mangold, 1987; Von Boletzky, 1971; 1984).

In decapods the ovum is surrounded by a chorionic membrane produced by the follicle cells of the ovary (Von Boletzky, 1987a). At the moment of spawning the eggs are first surrounded by jelly from the oviductal glands (Fioroni, 1978). The nidamental glands provide further envelopes (Jecklin, 1934, Lemaire, 1971; Tompsett, 1939). In sepiids and many teuthoids, like *L.vulgaris* and *L.forbesi*, the spermatozoa, stored in the buccal pouch, have to cross the nidamental jelly as the eggs (or egg strings) pass along the storage site. Therefore, freshly laid eggs are always soft and gelatinous (Von Boletzky, 1983; 1987a).

Eggs

All sepioids appear to lay the eggs one by one (Mangold, 1987). In *S.officinalis* the spirally coiled envelopes are stained with black ink (Bott, 1938; Grimpe, 1926; Schroder, 1966; Von Boletzky, 1983). Due to the expanding perivitelline fluid during embryonic development (De Leersnyder and Lemaire, 1972) the envelope finally reaches a maximum diameter of 1 5 cm (Fioroni, 1978; Von Boletzky, 1983). Especially at the end of embryonic development clusters of *Sepia* eggs resemble dark wine grapes.

The myopsid squids lay strings of eggs that are attached to a substrate in large masses (Mangold-Wirz, 1963) A number of eggs (about 90 in *L.vulgaris*) is embedded in oviductal jelly, which in turn is surrounded by layers of nidamental material in the form of a spiral (Fioroni, 1978; Mangold, 1987). The details of how this spiral arrangement is made by the female are not yet fully understood. An excellent description of egg strings with different coatings is given by Jecklin (1934). The spawns of *L.vulgaris* and *L.forbesi* appear very similar, but in 1928 Naef already drew attention to the fact that the egg masses as well as individual eggs and embryos are markedly larger in *L forbesi*. This is confirmed by more recent observations by Von Boletzky (1987b).

Embryonic development

The embryonic phase of the cephalopod life cycle has been studied by numerous zoologists since the middle of the nineteenth century (for reviews see Naef, 1923; 1928; Arnold, 1971; Fioroni, 1978; Von Boletzky, 1987a). For embryological studies, cephalopod eggs have always been particularly interesting, because they are very large and permit observation of many details of embryogenesis in vivo, at low microscopic magnification (Marthy, 1972; Von Boletzky, 1987a). The first description of cephalopod development was by Naef (1928) for *L.vulgaris*. In more recent years it was followed by many other illustrated stage descriptions (Lemaire, 1970 for *S.officinalis*; Sewaga et al., 1988 for *L.forbesi* and Arnold, 1965a for *Loligo pealei*).

In cephalopods the duration of embryonic development is always temperature dependent and may vary between 31 (21.4 °C) and more than 69 days (15.9 °C) in *Sepia* (Fioroni, 1978; Lemaire, 1970, Lemaire 1971; Richard, 1971) and between 18 (23.5 °C) and 45 days (14.0 °C) in *L.vulgaris* (Mangold-Wirz, 1963; Von Boletzky, 1987a).

The pre-organogenetic phase of cephalopod development is characterized by 1) the early fixation of bilateral symmetry, which becomes particularly distinct with the formation of the first cleavage furrow, and 2) the meroblastic cleavage, which leaves the large yolk mass in a syncytial state, while the blastomeres constitute a cap-shaped "disco-blastula" covering the animal pole of the egg. There are no traces of the ancestral mode of spiral cleavage typical of molluscs (Von Boletzky, 1987a).

In the cephalopod embryo the yolk is covered by a special syncytial layer (Lankester, 1875) which is digestive in function and morphogenetic in significance (Arnold, 1965b; 1971). During and after the formation of the outer yolk sac syncytium, the organ rudiments of the embryo proper appear. At first, "areas" representing future organ complexes can be recognized, subsequently the individual rudiments become distinct. The actual shaping of the embryonic body starts with a series of folds leading to the formation of the shell sac in the mantle, the eye and the statocyst vesicles, and the stomodaeum. These processes are enhanced by a progressive radial contraction

of the entire embryonic cap. Throughout progressive organogenesis the syncytium of the outer yolk sac plays the role of an embryonic heart (Von Boletzky, 1987a).

The inner yolk sac is progressively reduced under the pressure of the developing organs surrounding it. At the end of the period of radial concentration, the embryonic body presents the typical architecture of a cephalopod: a mantle sac enclosing the cavity in which a pair of rudimentary gills and the site of the future anal opening are hidden, the funnel complex closing the mantle cavity anteriorly, and the head marked by very large eyes and the ring of arms surrounding the mouth (Von Boletzky, 1987a; Arnold, 1971, Fioroni 1978). Towards the end of embryonic development the similarity between the embryo and the adult increases. In Fig. 1.1 advanced embryonic development of *L.vulgaris* is visualized.

Hatching

As in many other invertebrates embryonic development in cephalopods ends with the hatching process (for a phylogenetically comparative study see Denucé, 1984). Development in cephalopods is direct and the embryos finally hatch as miniatures of the adult stage (Roper et al , 1984). The hatching gland of decapod cephalopods, the so-called organ of Hoyle (Hoyle, 1889) synthesizes an enzyme which at the moment of hatching is released to dissolve the surrounding envelopes locally (Denucé and Formisano, 1982, Fioroni, 1978; Vayssière, 1910; Von Boletzky, 1989b; Von Orelli, 1959, Wintrebert, 1928). The ciliary bands of the mantle integument provide most of the locomotory effort during hatching when the animal crosses the gelatinous envelopes of the egg mass (Von Boletzky, 1979a; 1980; 1982a).

Up to the present very little is known about environmental factors controlling hatching in decapod cephalopods. Only a few studies are dealing with the regulation of hatching. According to Marthy et al. (1976) and Weischer and Marthy (1983), a substance present in the perivitelline fluid would prevent premature hatching by virtue of its tranquillising effect on the embryonic ciliature. The substance is likely to be a polypeptide or a protein of about 60,000 dalton. The mechanism by which this inhibition is lifted at hatching remains unexplained. In 1968 Atkinson and Granholm found that a component of the egg string jelly of *L.pealei* also inhibits ciliary activity and it was concluded that the nidamental gland was the source of the ciliary immobilizing factor (Atkinson, 1973). Investigations of Denucé and D'Aniello (1984) indicated that hatching can be induced experimentally in mature squid embryos by a heat-stable, rapidly diffusing, low molecular weight constituent (< 1.000 dalton) present in the adult brain (see also Denuce, 1984).

Aims of the present study

In the present study some relevant aspects of hatching in decapod cephalopod embryos were investigated. One could ask whether it is really so important to study the hatching process in cephalopods. Those who study this process have been in the first place impressed by the fact that hatching is a critical moment in the life of a developing organism, during which the micro-environment around the embryo is being replaced in a rather abrupt way by direct contact with the outer world There are a number of indications spread in the literature that this breakthrough is accompanied by drastic biochemical and structural changes (Backstrom, 1959, 1969, Denucé, 1984; Murison, 1969; Shakley et al., 1974).

Second, lately cephalopods are becoming increasingly important for fisheries (Roper et al., 1984), pharmacological studies and the biological sciences in general (Marthy, 1972). The utilization of cephalopods is manyfold, ranging from fresh consumption (eaten raw as "sashimi" in Japan) to various types of processing (dried, canned, frozen, reduced to fishmeal etc.) Their high protein and low fat content make cephalopods an important element in the diet of human populations (Roper et al., 1984). In 1981 the reported catch of cephalopods totalled 1 304.000 metric tons of which 71 8% was accounted for by neritic and oceanic squids (*e.g Todarodes, Loligo, Illex*), 13.6% by cuttlefish (*Sepia, Sepiella* and allied genera) and about 14.6% by octopuses (mainly *Octopus* and *Eledone*). More than half of the total catch was taken in the northwest and northeast Pacific and the northeast and northwest Atlantic. Most of this catch (around 700.000

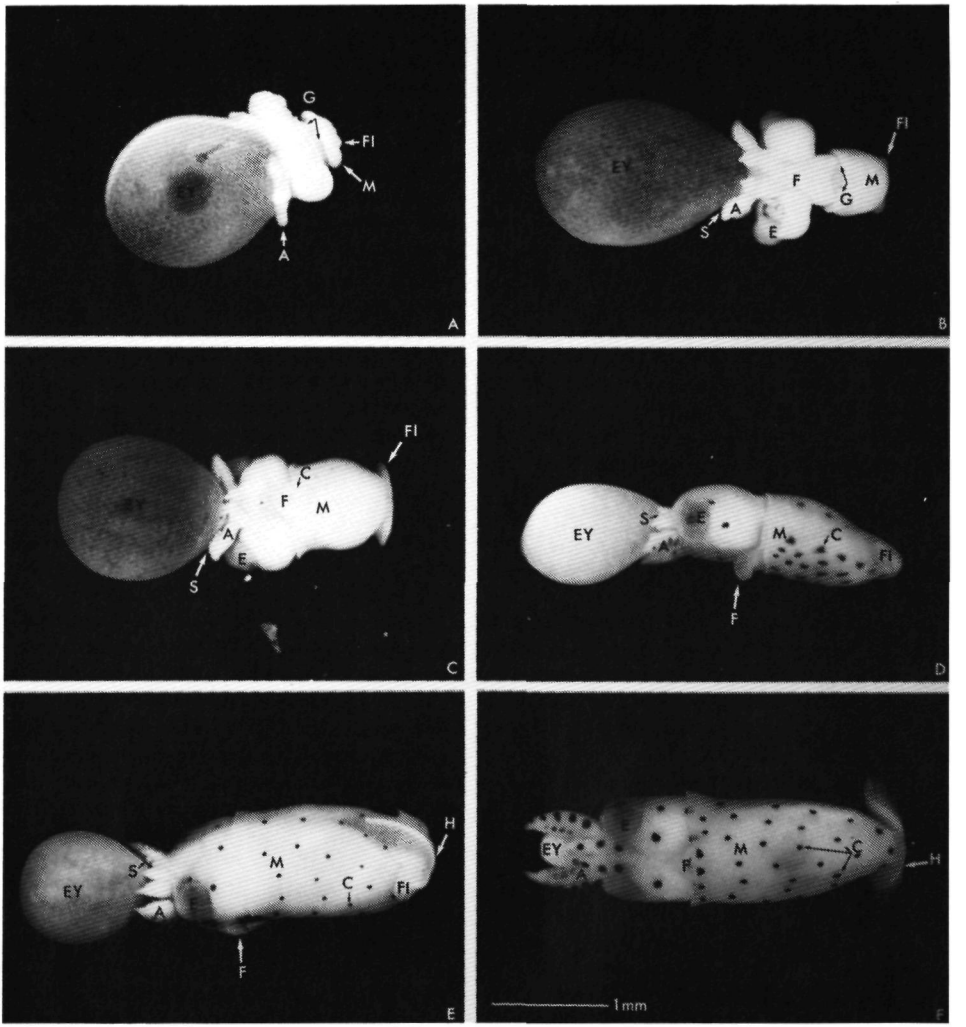

Fig. 1.1. Late embryonic development of *Loligo vulgaris*. Stages according to Naef (1928). (a) stage XI (dorsal view). The gills, arms, funnel folds and mantle stick out above the rest of the embryo. The anterior and posterior funnel folds fuse together. The shell sac closes completely, and fin folds develop on the broadening mantle. The optic lobes are prominent. (b) stage XII-XIII (ventral view). The mantle almost completely covers the anal papilla, gills and the posterior portion of the funnel, leaving a small opening. The funnel tube is closed. A division marks the internal and external yolk. (c) stage XIV (ventral view). The mantle completely covers the posterior margin of the funnel. The first ventral chromatophores display an orange pigmentation. The suckers on the arms are prominent. (d) stage XVI-XVII (lateral view). Dark red chromatophores are visible. (e) stage XVIII (dorso-lateral view). The external yolk sac has approximately the same size as the head of the embryo and Hoyle's organ (hatching gland) can be discerned. The dorsal chromatophores are evident, but few in number compared to stage XX. (f) stage XX (hatching stage) (ventral view). The external yolk sac is almost completely absorbed. (A) arms, (C) chromatophore, (E) eye, (EY) external yolk sac, (F) funnel, (Fl) fin, (G) gills, (H) Hoyle's organ, (M) mantle, (S) sucker.

metric tons) was taken by Japanese vessels. The role of cephalopods in the ecosystems seems to be that of subdominant predators. It is obvious that if cephalopods become depleted because of heavy fishing this will lead to dramatic changes in the marine ecological system [which has been observed in the Gulf of Thailand (Roper et al., 1984)]. Recently, attempts were made to rear cephalopods in aquaculture but many problems have to be overcome (Choe, 1966; Hanlon et al., 1987; Laroe, 1971; Robaina, 1979; Von Boletzky, 1974). Because hatching is a critical moment in the life of a developing organism (Denucé, 1984) and can affect the survival of a species in many ways, an inquiry into the way hatching is controlled and carried out in cephalopods is therefore not only of scientific interest but will eventually provide basic elements for aquaculture and marine environmental policy.

The study of hatching in cephalopods depends of course upon the availability of living material. In this respect, *Loligo* could pose a problem, as eggs in the North Sea can only be obtained during a very short period. Experimental work is therefore limited. The results of the present study could only be obtained by splitting up the work in two main parts: structural and biochemical investigations on preserved material and experimental work with living material. During the period eggs were available, three environmental factors probably controlling or affecting late embryonic development and hatching (copper, light and salinity) were experimentally investigated (Part two of this thesis). Because structural studies on the organ of Hoyle of *L.vulgaris* and *S.officinalis* were rather old (Hoyle, 1889; Yung Ko Ching, 1930; Von Orelli, 1959) or inexistent as in the case of *L.forbesi*, and biochemical studies on the hatching enzyme were lacking, material collected in the same period was either fixed or frozen for the purpose of structural and biochemical studies. The results of these investigations, carried out during the period live biological material was not available, are combined in the first part of this thesis.

PART I: Structural and biochemical aspects

A SCANNING ELECTRON MICROSCOPE STUDY OF ECTODERMAL DIFFERENTIATIONS IN THE CAUDAL MANTLE EPITHELIUM OF EMBRYOS AND JUVENILES OF *LOLIGO VULGARIS, LOLIGO FORBESI* AND *SEPIA OFFICINALIS**

ABSTRACT The mantle epithelium of embryos and early juveniles of the squids *Loligo vulgaris* and *Loligo forbesi* and the cuttlefish *Sepia officinalis* was studied, using scanning electron microscopy In embryos of *L.vulgaris* and *L.forbesi*, some hitherto undescribed epidermal structures were found. They are missing in *S officinalis* embryos. These socalled "extruding structures" are located near Hoyle's organ and first appear in developmental stage XIII of Naef. At the same embryonic stage, Hoyle's organ starts to differentiate and "uniform-type" ciliated cells become visible in the epidermis of both *L.vulgaris* and *L.forbesi* Directly after hatching the epidermis of the species examined starts to slough off and finally the extruding structures, Hoyle's organ and both types of ciliated cells of the mantle epithelium disappear The function of the extruding structures remains obscure

INTRODUCTION

The epidermis of decapod cephalopod embryos shows various ectodermal differentiations. Although easily distinguishable, some have been hardly investigated thus far

Most striking among the epidermal differentiations are the ciliated cells which first appear in stage IX (Naef) embryos of *Loligo pealei* In total, four types of ciliated cells can be discerned in this species (Arnold and Williams-Arnold, 1980; Sundermann-Meister, 1978)

In advanced embryonic stages a patch of modified ectodermal cells, arranged in the form of an anchor, appears in decapod cephalopod species. This organ of Hoyle (Hoyle, 1889) is situated towards the posterior aspect of the body and probably produces an enzyme which locally digests the chorion, thus enabling the embryo to hatch (Jecklin, 1934; Denuce and Formisano, 1982).

In embryos of *Loligo vulgaris* (European squid), the cells of Hoyle's organ start to differentiate at stage X (Naef). At stage XII most cells are already filled with secretory granules (Meister, 1972) The hatching gland cells in *Sepia officinalis* (common cuttlefish) embryos differentiate earlier: the cells are already found at stage IX (Yung Ko Ching, 1930)

At the end of embryonic development the epidermis of *S.officinalis* and *L.vulgaris* consists mainly of mucous cells with large vacuoles (Fioroni, 1963). The skin of squid functions as an organ of secretion (Kisch, 1951a)

The present chapter describes the ciliated cells in the mantle epithelium and Hoyle's organ in embryos of *L vulgaris*, *L forbesi* and *S officinalis* in the course of embryonic development and at various times after hatching, using scanning electron microscopy. The appearance of these ectodermal differentiations and of hitherto undescribed extruding structures is compared

* Chapter 2 is the modified version of Pauli W P Denucé J M (1990) A scanning electron microscope study of ectodermal differentiations in the caudal mantle epithelium of embryos and juveniles of *Loligo vulgaris Loligo forbesi* and *Sepia officinalis* Inv Rep Dev 17 247 255

MATERIALS AND METHODS

The study was carried out with embryos of the squids, *Loligo vulgaris* and *Loligo forbesi* and the cuttlefish, *Sepia officinalis* Eggs of *L.forbesi* collected in the English Channel during November 1988 were sent to the Delta Institute for Hydrobiological Research at Yerseke (The Netherlands) where they were kept in tanks provided with running sea water coming directly from the Oosterschelde (salinity 30 ‰) In total 3 different batches were used.

Spawns of *L.vulgaris* and *S officinalis* were collected in the Oosterschelde from April till June 1988 and kept in an outdoor aquarium (1500 l) provided with circulating Oosterschelde water. Seven spawns of *L vulgaris* and 3 spawns of *S.officinalis* were used in the experiment

At least 15 embryos at various stages of embryonic development were investigated per species. *S.officinalis* embryos were staged according to Lemaire (1970); for *L vulgaris* and *L forbesi* embryos the developmental table of Naef (1923, 1928) was used.

As soon as the embryos of *L.vulgaris* and *L forbesi* reached the hatching stage (stage XX), several strings were removed from the aquarium and placed in a cuvette (10 x 10 x 6 cm) filled with sea water The cuvette was thereupon kept in complete darkness for 2 min. During this dark period about 80% of the embryos of both species hatched (Chapter 8 of this thesis).

After the dark-shock 20 embryos were immediately removed from the cuvette and fixed. The strings with the embryos that had not hatched were discarded. Early juveniles were collected 10, 20, 30 min and 1, 3, 4, 6, 12 and 24 hrs following the dark-shock.

Every morning juveniles of *S.officinalis* were collected from the outdoor aquarium. These embryos had hatched the night before and were not older than 10 hrs at the time of collection.

All the embryonic material was fixed in Hollande's fixative (Gurr, 1962) for 24 hrs, followed by dehydration through a graded series of ethanol and finally kept in absolute ethanol for at least 12 hrs. The material was then critical point-dried with CO_2. The embryos were mounted on metal stubs, sputter-coated with gold and finally examined with a Jeol JSM-T3000 scanning electron microscope For photographic recording Agfapan Professional 100 film was used.

RESULTS

The fate of the ectodermal differentiations in the two loliginid species is summarized in Table 2 I.

Ciliated cells

In stage IX embryos of *L.vulgaris* and *L.forbesi*, cilia appear on the future ventral surface of the mantle edge. The ciliated cells are large, flattened and separated from each other by non-ciliated cells. These so-called paddle-type ciliated cells are mainly found on the lateral sides of the mantle The fin primordia, visible as two thickenings on both sides of the mantle, are devoid of cilia (Fig 2 1)

In stage XIII *Loligo* embryos, another ciliated cell type appears on the mantle surface (Fig. 2.2a). These so-called uniform-type ciliated cells are elongated and form "lines" or "bandes ciliaires" (Von Boletzky, 1980) on the mantle epithelium as they contact each other (Fig. 2 2d). The lines of these cilia radiate from the future site of Hoyle's organ. In young cells cilia of unequal length can be found

In stage XVIII and XX *Loligo* embryos, the uniform-type cilia are clearly visible as long rows on the dorsal and ventral part of the mantle (Figs 2.2b and d) The epithelium of the fins remains devoid of ciliated cells, except in the middle and near Hoyle's organ (Fig 2 2d) Both types of ciliated cells also occur in *Sepia* embryos, but in this species the lines of uniform-type cilia are not as well developed as in *Loligo* (Fig. 2 3c).

After hatching, changes occur in the epithelium of the mantle of all three species (Figs. 2 3, 2 4, 2 5 and 2.6). Between 1 and 2 hrs after hatching, the ciliated cells in the mantle epithelium of *L.vulgaris* and *L.forbesi* start to degenerate (Figs 2.4c and d). The paddle-type ciliated cells seem to disappear before the uniform-type ciliated cells. They were never found in juveniles older than 1 hr The uniform-type cilia however, were still visible 6 hrs after hatching, but only on the dorsal part of the mantle or in tiny clusters near Hoyle's organ (Fig 2.4f)

Table 2.I. Fate of ciliated cells, Hoyle's organ and extruding hemispheric structures in embryos of *Loligo vulgaris* and *Loligo forbesi* before and after hatching

stage X	paddle-type ciliated cells appear		
stage XIII	uniform-type ciliated cells appear	Hoyle's organ becomes evident	extruding structures appear
stage XX			difference flat and extruding structures most pronounced
directly after hatching		apical parts of Hoyle cells disintegrate	
1 - 2 hrs	degeneration of ciliated cells	cells of Hoyle's organ have disappeared	
12 hrs	most cilia have disappeared		extruding structures disintegrate

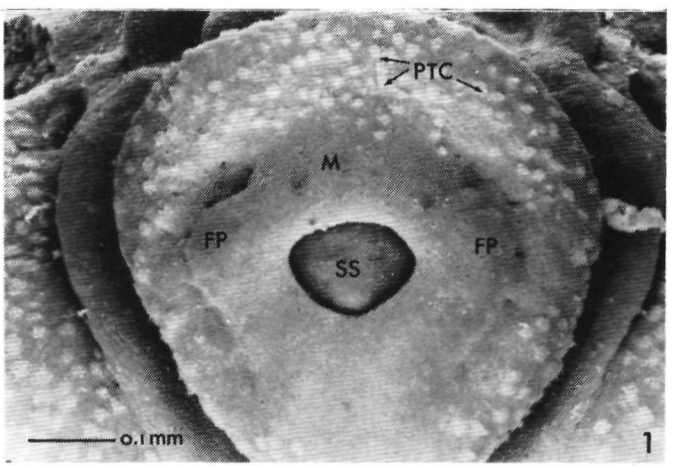

Fig. 2.1. *Loligo vulgaris.* Mantle of a stage IX embryo during closure of the shell sac. Paddle-type cilia have developed on the future ventral surface and on the sides of the mantle. In this embryonic stage neither uniform-type cilia, nor Hoyle's organ have developed. No extruding structures have appeared yet. M = mantle; PTC = paddle-type ciliated cells; SS = shell sac; FP = fin primordia.

In early juveniles of *L.vulgaris* and *L.forbesi*, most cilia have disappeared 12 hrs after hatching (Figs. 2.4g and h). Instead, large holes were found in the epithelium of the mantle (Fig. 2.4h). In *Sepia* all ciliated cells degenerate more rapidly than in the loliginids.

After 24 hrs post-hatching another change in the epithelium of the mantle occurs in *L.vulgaris* and *L.forbesi*. Not only do the ciliated cells disappear but the mantle becomes covered by clusters of dead cells (Fig. 2.6) and finally the whole mantle epithelium is sloughed off.

Hoyle's organ

The formation of Hoyle's organ becomes evident in stage XIII embryos of *L.vulgaris* and *L.forbesi*. In contrast to the conspicuous uniform-type ciliated cells, the cells of Hoyle's organ are not ciliated so that the hatching gland becomes prominent as a non-ciliated, T-shaped aggregation of cells (Fig. 2.2a). The apical surface of the Hoyle cells is still intact but as the embryo develops it becomes more irregular (Fig. 2.2b).

Immediately after hatching a sudden change occurs: the apical part of the cells disintegrates and large holes become visible among the vestiges of the glandular cells, indicating the sites of release of the hatching enzyme. These holes were found in all regions of the gland but mainly in the centre (Figs. 2.4a and 2.5a). About 1 hr after hatching, most holes are no longer visible as they are covered with an irregularly shaped material that probably is of cellular origin (Fig. 2.4c).

Finally, Hoyle's organ undergoes regressive changes, as is clearly visible between 1 and 2 hrs after hatching (Figs. 2.4c and d). By then most cells have disappeared but the vestiges of the anchor-shaped gland can still be discerned.

Extruding hemispheric structures

In stage XIII embryos of both *L.vulgaris* and *L.forbesi* a number of non-ciliated structures appear at the intersection of the three branches of Hoyle's organ (Fig. 2.2a). They are not found in any other part of the embryonic epithelium. In stage XIII the structures are rather large, flattened and not

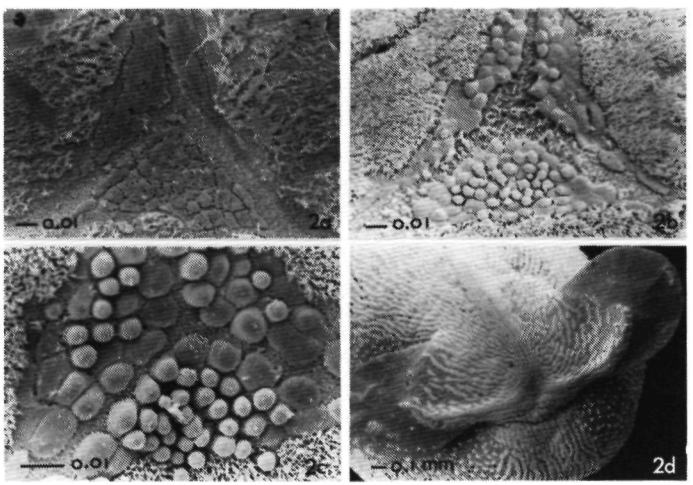

Fig. 2.2. *Loligo vulgaris.* Hoyle's organ, uniform-type ciliated cells and extruding structures at stage Naef XIII(a), XVIII(b) and XX(c and d) embryos. Hoyle's organ is the anchor-shaped organ surrounded by uniform-type cilia. The extruding structures are found at the intersection of the three branches of Hoyle's organ. In stage XIII embryos the extruding structures are flattened and therefore not sharply outlined (a). In stage XX embryos, the difference between the flat and hemispheric extruding structures is most pronounced (c).

sharply outlined. In the course of development they project above the surface, but strikingly the structures in the immediate vicinity of Hoyle's organ remain flattened (Fig. 2.2b). The difference between flat and extruding structures becomes most pronounced at stage XX (Fig. 2.2c), although some intermediate stages can still be discerned. At this stage most flat structures appear only in the immediate vicinity of Hoyle's organ.

Although the extruding structures do not increase in number, their surface undergoes clear changes: the flattened structures measure $6.0 \pm 2.0\ \mu m^2$, whereas the surface area of the extruding structures is $2.0 \pm 1.0\ \mu m^2$. Following hatching the extruding as well as the flat structures undergo considerable changes. Already 10 minutes (Fig. 2.4b) after hatching the structures in the proximity of Hoyle's organ are less flat than in pre-hatch stage XX (Fig. 2.2c). One hour after hatching it seems that the degenerating Hoyle cells are becoming surrounded by extruding structures only (Fig. 2.4c). Probably the latter have originated from the flat structures.

Between 3 and 6 hrs after hatching, most ciliated cells have disappeared, but the hemispheric structures are still clearly visible (Figs. 2.4e and f). Only 12 hrs after hatching it becomes evident that these structures are also degenerating. In some cases there will be very few left (Fig. 2.4g), but in most juveniles the hemispheric structures have vanished after 24 hrs.

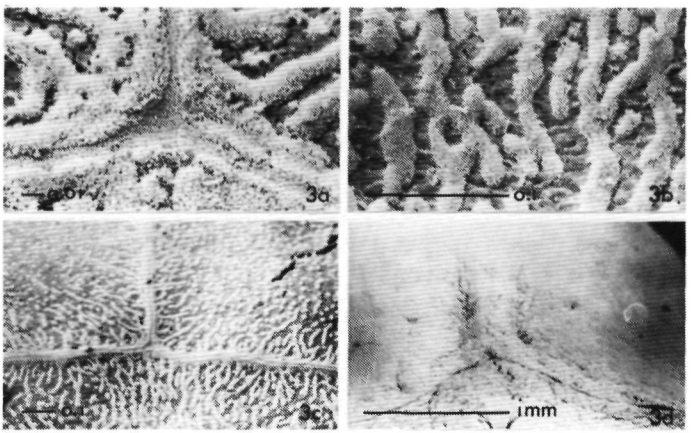

Fig. 2.3. *Sepia officinalis.* Hoyle's organ and uniform-type ciliated cells in a stage 30 (Lemaire, 1970) embryo (a and c). The anterior-posterior "lines", formed by the uniform-type ciliated cells, are not as well developed as in *L.vulgaris* and *L.forbesi* embryos (b)(compare figs. 2.2c and d). About 24 hrs after hatching all uniform-type ciliated cells of *S.officinalis* have disappeared (d). The remnants of Hoyle's organ can still be discerned as a rift in the form of an anchor.

DISCUSSION

The appearance of ciliated cells in the integument of embryos is not restricted to cephalopods. In the embryonic epidermis of *Rana pipiens*, ciliated cells appear during the neural plate stage. As in decapod cephalopod embryos, these cells degenerate after hatching (Kessel et al., 1974). Ciliated cells are also found in embryos of *Xenopus laevis* (Smith et al., 1976) and *Ambystoma mexicanum* (Billett and Courtenay, 1973; Landström, 1977). According to Kessel et al. (1974), embryonic ciliated cells are important for respiration and the movement of mucus over the embryo.

In *L.pealei* embryos, ciliated cells first appear at Naef stage VIII on the external yolk sac (Arnold

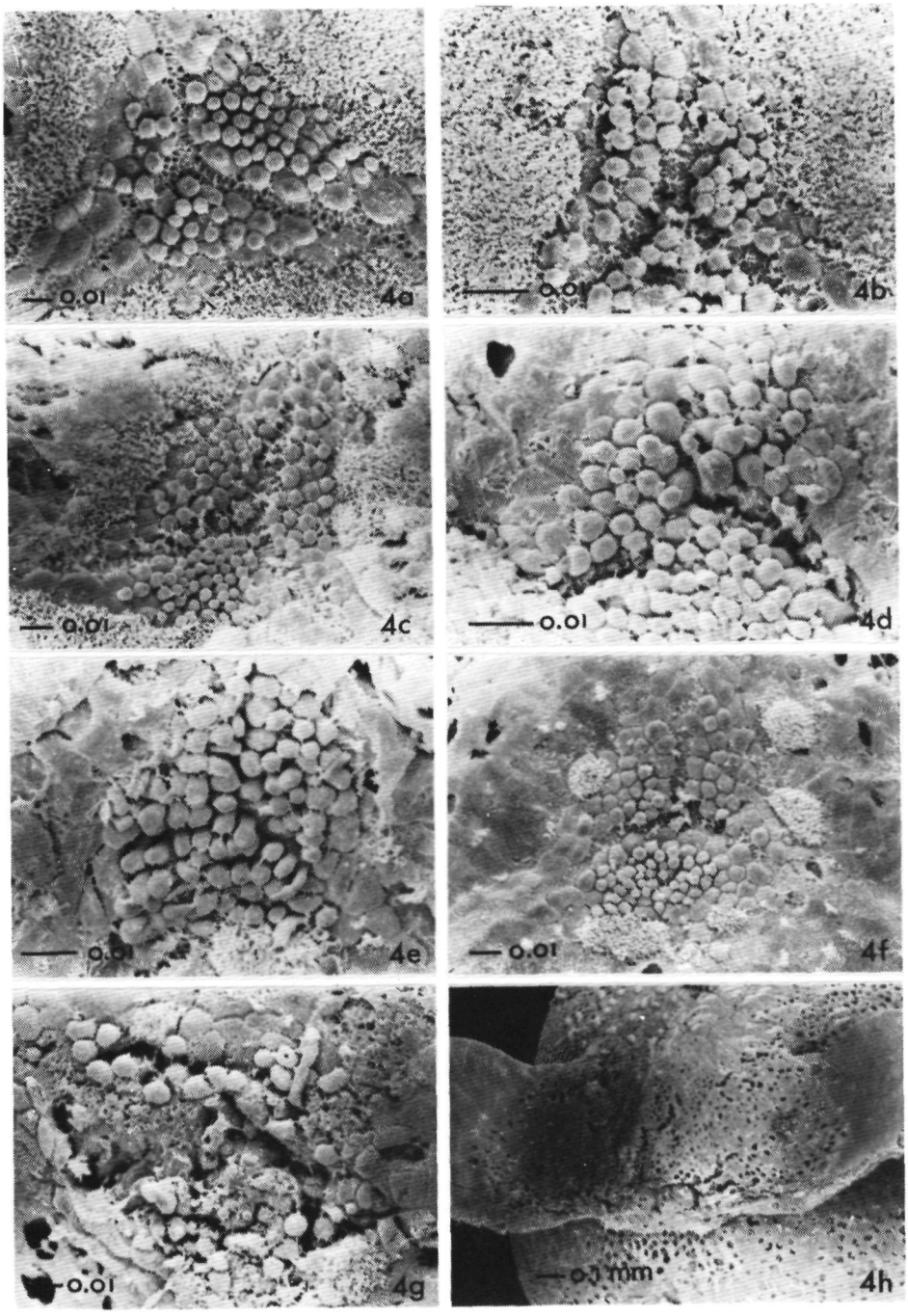

Fig. 2.4. *Loligo forbesi.* Hoyle's organ, uniform-type ciliated cells and extruding structures; immediately (a), 10 min (b), 1 hr (c), 2 hrs (d), 3 hrs (e), 6 hrs (f), and 12 hrs (g and h) after hatching.

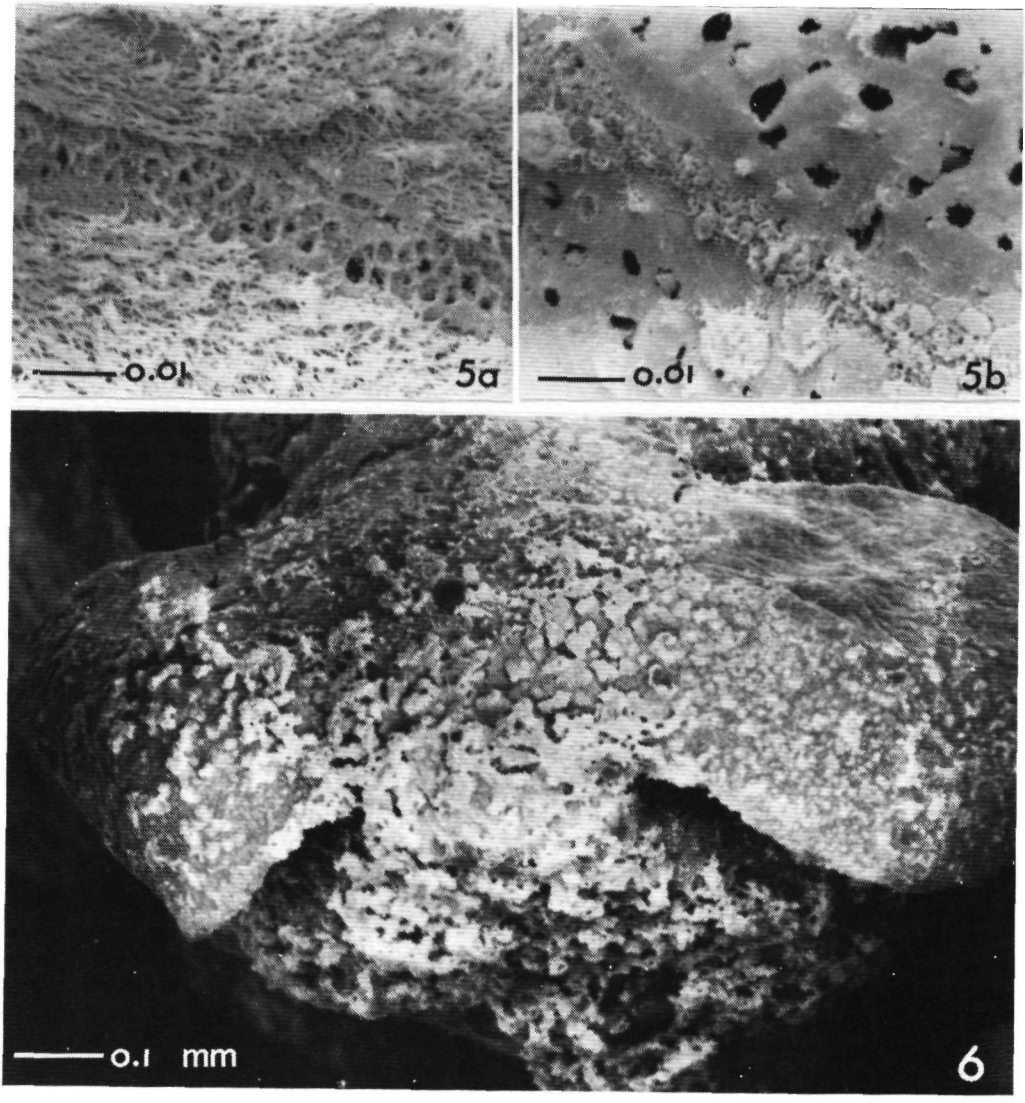

Fig. 2.5. *Loligo forbesi.* One of the lateral branches of Hoyle's organ immediately (a) and 6 hrs (b) after hatching.

Fig. 2.6. *Loligo forbesi.* Dorsal aspect of an early juvenile 24 hrs after hatching. The mantle and fins are covered by clusters of discharged cells.

and Williams-Arnold, 1980). In the mantle epithelium, paddle-type ciliated cells are first found at stage IX and uniform-type ciliated cells at stage XIII (Arnold and Williams-Arnold, 1980). These results are in agreement with our observations on embryos of *L vulgaris* According to Von Boletzky (1980) the formation of paddle- or club-shaped ciliary tips of the paddle-type cilia is an artefact caused by tissue fixation.

Degeneration of mantle epithelium of cephalopod embryos after hatching was first observed by Faussek (1901) His findings were confirmed by Yung Ko Ching (1930) and Ranzi (1931) In contrast, Fioroni (1963) found that during hatching in *S.officinalis*, the mucous cells are practically emptied, but that no degeneration of the epidermis takes place. The presence of active and pycnotic nuclei, and vacuoles of different sizes, was interpreted as an indication of epidermal regeneration. Our observations, however, suggest that after hatching all ciliated cells, Hoyle's organ and the extruding structures disappear An explanation can be found in partial epidermal degeneration: in *S officinalis* embryos probably only the ciliated cells, Hoyle's organ and the extruding structures disintegrate after hatching, while the mucous cells are only emptied

The cells of Hoyle's organ produce a hatching enzyme (Denucé and Formisano, 1982). Cells in the hatching gland of most decapod cephalopods, like *Sepiella japonica*, closely resemble the hatching gland cells of teleosts and amphibians. The secretory granules are similar in size, electron density and intracellular distribution (Matsumo and Ouji, 1988). The nucleus is located near the basis of the cell as in the hatching gland of the teleosts *Brachydanio rerio*, *Danio malabaricus*, *Moenkhausia oligolepis* and *Barbus schuberti* (Willemse and Denucé, 1973). The hatching enzyme is contained within granules that arise from the Golgi apparatus (Wintrebert, 1928) Hoyle's organ has no function after hatching so its degeneration is not surprising.

The function of the ciliated cells is less evident. According to Arnold and Williams-Arnold (1980), the most important feature of the uniform-type cells is their metachronal beat. The alignment of the cells and the synchrony of the beat seem quite effective in moving around large volumes of fluid, causing movement of the embryo. Another hypothesis regarding the function of the ciliated cells has been advanced by Von Boletzky (1982a) According to his observations on embryos of *L vulgaris*, the ciliary bands of the mantle integument provide most of the locomotory effect during hatching when the animal traverses the gelatinous envelopes of the egg mass In *Sepia* these ciliated bands are less developed because the embryo has to pass through a thinner egg capsule during hatching (Von Boletzky, 1978, 1982a) In the incirrate octopods, the integument of the embryo is entirely devoid of cilia. A set of special integumental structures, the so-called Kolliker organs, is used as auxiliary hatching equipment, when the young animal leaves the chorion, which is the only envelope surrounding incirrate octopod eggs (Fioroni, 1963) These hypotheses are in accordance with the fact that in all species examined, ciliated cells disappear after hatching.

The function of the extruding hemispheric structures remains unexplained Their location on the mantle surface, in the vicinity of Hoyle's organ and their disappearance after hatching suggest that they might be involved in the hatching process. In support of this assumption we have also noticed that hatching of *L.vulgaris* and *L forbesi* is preceded by a stereotype pattern of behaviour consisting of repeated bumping of the abdomen against the chorion with the stretched mantle tip. It is evident that in this process the extruding structures are the first to come into contact with the chorion.

The extruding structures were first mentioned and figured by Hulet (1982) who thought they were enzyme-filled cells. But these structures are clearly distinguishable from Hoyle's organ. Immediately after hatching holes appear in the cells of this gland, indicating the place where the hatching enzyme is released, whereas such holes have never been observed in the extruding structures Furthermore, the hatching gland and the extruding structures disappear at different times

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HEMISPHERIC-APEX CELLS IN THE MANTLE EPITHELIUM OF *LOLIGO VULGARIS* AND *LOLIGO FORBESI* HATCHLINGS AND JUVENILES: A TRANSMISSION ELECTRON MICROSCOPE STUDY*

ABSTRACT. Hemispheric-apex cells (HC), which are situated at the intersection of the three branches of Hoyle's organ in *Loligo vulgaris* and *Loligo forbesi* embryos, were investigated using light and transmission electron microscopy. The surface membrane of the HC exhibits many microvilli. Desmosomes between HC were distributed in patches along the plasma membrane. These desmosomes were also found between HC and the hatching enzyme producing cells of Hoyle's organ (CHO) and between neighbouring CHO. The area below the most apical desmosomes showed many cellular interdigitations. One (or several) small Golgi zones and many mitochondria were found in the cell centre. Mitochondria were also abundant in the most apical part of the cell. In the HC the nucleus is situated near the cell centre. The shape of the central part of the cell usually corresponds grossly to the nuclear form. Endoplasmic reticulum was found near the nucleus and in the lowermost part of the cell, but was never as abundant as in the CHO. In the most apical part of the HC many oval vesicles were found containing electron lucent material. Ultrathin sagittal sections showed that the tapering proximal part of the HC, directed toward the underlying tissue, may end in a T-shaped form. As the HC and CHO degenerate after hatching, it is concluded that both structures are transient embryonic cells.

INTRODUCTION

During the embryonic phase animals are covered by protective structures of different architecture and origin (Schoots et al., 1983a). In the decapod cephalopod species *Loligo vulgaris* (European squid) and *Loligo forbesi* (veined squid), the embryo is surrounded by a chorionic membrane produced by the follicle of the ovary. The oviducal gland surrounds the chorion with some jelly. After leaving the oviduct, the eggs are wrapped in additional protective envelopes (Jecklin, 1934). The latter are produced by the nidamental glands, which lie at the ventral surface of the visceral mass within the mantle cavity (Von Boletzky, 1987a).

In cephalopods the embryonic phase ends with the hatching process (Fioroni, 1978). As in many other invertebrates, the hatching enzyme of decapod cephalopods is produced and stored in a transient specialized gland (Hibbard, 1937; Ishida, 1971; Denucé and Formisano, 1982; Denucé, 1984; Matsuno and Ouji, 1988). The cells of this so-called organ of Hoyle form a trifid tract situated towards the posterior end of the body: one branch in the mid-dorsal line, the others on the dorsal surface of each fin, reaching about midway between its base and its free margin (Hoyle, 1889; Yung Ko Ching, 1930).

Recently, Arnold and Singley (1989) and Paulij and Denucé (Chapter 2 of this thesis) described

Chapter 3 is the modified version of Paulij, W P , Kallen, J L , Denucé, J M (1991) Hemispheric-apex cells in the mantle epithelium of *Loligo vulgaris* and *Loligo forbesi* hatchlings and juveniles a transmission electron microscope study Inv Rep Dev 19 in press

a number of extruding structures which appear at the intersection of the three branches of Hoyle's organ. These structures were first mentioned and depicted by Hulet (1982) who thought that they were enzyme filled cells. More recent investigations, however, indicated that the extruding structures are clearly distinguishable from the enzyme producing cells of Hoyle's organ. On the other hand, their location on the mantle surface, in the vicinity of Hoyle's organ, and their disappearance after hatching suggest that they are involved in the hatching process.

The present study describes the hemispheric-apex cells (HC) which form the extruding structures, at the end of embryonic development, and at different times after hatching, using light microscopy and transmission electron microscopy. The morphology and appearance of these cells are compared with the enzyme producing cells of Hoyle's organ (CHO).

MATERIALS AND METHODS

Biological material

Spawns of *Loligo vulgaris* attached to fishing nets in the Oosterschelde (South Western part of the Netherlands) were collected by fishermen during Spring 1988 and 1989. *Loligo forbesi* spawns were obtained from the English Channel during May, June and November 1988 and 1989 and were sent at very early developmental stages to the Delta Institute for Hydrobiological Research at Yerseke (The Netherlands). Spawns were divided into individual strings which were suspended vertically in an outdoor aquarium provided with running sea water (capacity 15 litres).

During development the condition and morphological features of the embryos were checked, and starting with stage XI (Naef 1923; 1928), embryos were taken out of the egg capsules and fixed for histological study as described below. As soon as the embryos reached stage XX (hatching stage), several strings were taken from the aquarium and placed for two minutes in complete darkness. During this dark-shock most embryos hatched (Chapter 8). The egg strings were removed from the cuvette and the juveniles were fixed immediately or 10, 60, 180 and 360 minutes after the onset of darkness.

Light microscopy

Embryos and juveniles were fixed in Hollande's fixative (Gurr, 1962) for 24 hrs The fixed material was dehydrated and embedded in paraffin according to conventional histological procedures. Sections (5 µm) were stained with haemalum-eosin (HE)

Transmission electron microscopy

Embryos and juveniles of both species were fixed in 2% glutaraldehyde in filtered (Millipore; 0.45 µm) sea water (Oosterschelde; salinity about 30 ‰.) for 1 hr on melting ice, followed by 1% OsO_4 in 0 1 M sodium cacodylate (pH 7.2, containing 0.8 M sucrose) for 1 hr on melting ice The specimens were dehydrated in a graded ethanol series and rinsed twice in propylene oxide for 30 min. Finally, the embryos were embedded in EPON 812.

Semithin sections (1 µm) were mounted on glass slides and stained with toluidine blue. Ultrathin sections were mounted on formvar/carbon-coated 50 mesh grids or uncoated 150 mesh grids and poststained with 2% aqueous uranyl acetate and lead citrate (Reynolds, 1963). The ultrathin sections were examined and photographed using a Philips 201 electron microscope.

RESULTS

Hemispheric-apex cells before hatching

A schematic drawing of the hemispheric-apex cells (HC), which appear at the intersection of the three branches of Hoyle's organ (Fig. 3.2a), is given in Fig 3 1. At developmental stage XX (Naef) the height of these cells, measured from the basal lamina to the tip of the microvilli, is 61 1 ± 3.5 µm.

The electron microscopic study shows that the surface membrane of the HC exhibits a multitude of fingerlike processes which will be referred to as "microvilli" (Fig. 3 6a). These structures are 1 3 ± 0.8 µm long with a diameter of 80 ± 20 nm and were also distinguishable in

HE stained sections (Figs. 3 2b and c). Inside the microvilli many microfilaments were counted (Fig. 3.6a). The microvilli considerably increase the surface area of the cell; in stage XX (Naef) embryos at least 36 microvilli µm ^{2} were found. The apical tips of the microvilli are frequently covered by a *cell coat* (Fig. 3.6a)

In the uppermost part of the HC many oval granules occur containing only lightly staining material. The same region also contains a number of round, non-staining vesicles (Fig. 3.5a).

Desmosomes are distributed in patches on the surface of the HC, in the region immediately below the microvillous extruding part (Fig. 3.3). They appear as juxtaposed disk-shaped electron dense structures on the cytoplasmic faces of the cell membranes of two neighbouring HC or

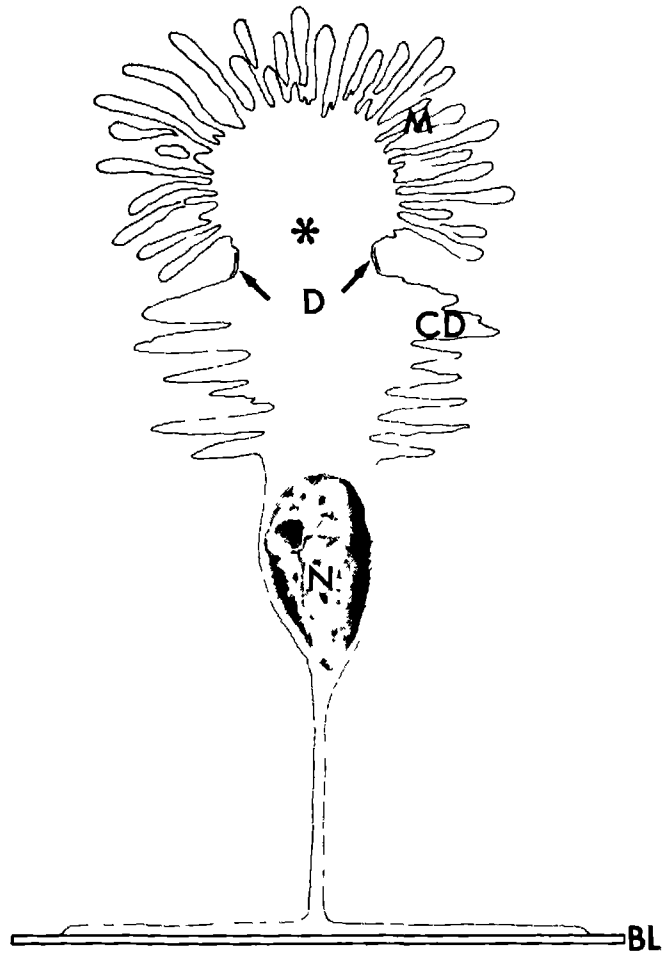

Fig. 3.1. A schematic drawing of the hemispheric-apex cells (HC), which appear at the intersection of the three branches of Hoyle's organ of *Loligo vulgaris* and *Loligo forbesi* embryos. BL = basal lamina, CD = cellular interdigitations, D = desmosome, M = microvilli, N = nucleus. * = exposed part of the HC

between HC and uniform-type ciliated cells (Fig. 3 3b). These regions are called attachment plaques or dense cytoplasmic plates (Junqueira and Carneiro, 1980) and consist essentially of a granular electron dense material, closely associated with the innermost surface of the cellular unit membrane Between the plasma membranes of HC desmosomes, fibrillar and granular structures were frequently observed (Fig. 3.3b). Desmosomes were also found between HC and the hatching enzyme producing cells of Hoyle's organ (CHO) and between neighbouring CHO.

The area below the most apical desmosome of the HC shows extensive cellular interdigitations (Fig. 3.4a), which increase the area of surface contact between the HC and probably contributes to the adhesion of these cells. HC are extremely cohesive. Cellular interdigitation is also found between the HC and the CHO (Fig. 3.4b).

In the HC one or more Golgi profiles and many mitochondria are found. The Golgi apparatus is rather small and appears in the central part of the cell as stacks of flat saccules with peripheral dilatations. The mitochondria occupy not only the central part but also the most apical, extruding part of the cell. At least 15 mitochondrial profiles per cell can be found.

In the HC the nucleus is always situated near the cell centre, 40.0 ± 4.0 µm from the basal lamina (Figs. 3.1 and 3.2a) The long axis of the pear-shaped nucleus is always parallel to the main axis of the cell. The shape of the central part of the cell usually grossly corresponds to the nuclear form (Fig. 3.2c). Endoplasmic reticulum occurs near the nucleus and in the lower part of the cell.

The basal part of the HC, extending toward underlying tissue, is tapering and ends in a T-shaped form as can be seen in ultrathin sagittal sections. Between the basal surface of CHO and HC and the underlying connective tissue, the basal lamina appears as a continuous layer of amorphous material (Fig. 3.4d).

Hemispheric-apex cells compared with the cells of Hoyle's organ

The HC appear very different from the more "prismatic" CHO (Figs. 3.2a,b and 3.5b). The CHO do not exhibit microvilli. In the CHO the rounded nucleus is always located near the base of the cell (Figs. 3.2a and b) and this region is extremely rich in granular endoplasmic reticulum (Fig. 3 4d). In the HC, on the contrary, the nucleus is located near the cell centre and endoplasmic reticulum is never as abundant as in the CHO.

In the CHO the Golgi apparatus is located in the supranuclear region of the cell. The remainder of the cytoplasm of these cells is filled with rounded, protein-rich secretory granules (Fig. 3.5b). Inside these granules dense and electron lucent regions can be discerned. The oval granules of the HC are only found in the most apical part of the cell (Fig. 3.5a) and contain only lightly staining material.

Hemispheric-apex cells and cells of Hoyle's organ after hatching

In figures 3 5a and b the apical part of the CHO and the HC at stage XX (Naef) are shown The CHO are completely filled with secretory granules (Fig. 3.5b), and the HC are also loaded (Fig. 3 5a) with material.

Immediately after hatching changes occur in both CHO and HC. In the CHO, the dense and electron lucent granules have partly vanished after releasing their product, *i.e.* the hatching enzyme, through multiple exocytosis (Fig. 3.5d). But not all enzyme containing granules are released at hatching. Besides empty holes (probably vacuoles) many secretory granules remain inside the cells (Fig. 3.5d; Fig. 3.2d)

In the HC two striking changes occur immediately after hatching: the apical part of the cells becomes less dense and the microvilli undergo degeneration (Figs. 3 5c and d). Small vesicles (diameter 150 ± 6 nm) with lucent contents appear between the microvilli (Figs. 3.5c,d and 3.6b). About 2 hrs after hatching larger vesicles are present near the base of the microvilli, while 6 hrs after hatching microvilli are degenerating or have disappeared completely (Figs 3.6c and d) After hatching cellular interdigitations become less extensive and small vesicles or holes are sometimes visible (Fig. 3 4c). About 30 minutes after hatching the nucleus of the HC has become pycnotic.

Changes very similar to those found in the HC were observed in the uniform-type ciliated cells. Six hrs after hatching the nucleus appears pycnotic and the cilia of the cells degenerate (Fig. 3.7).

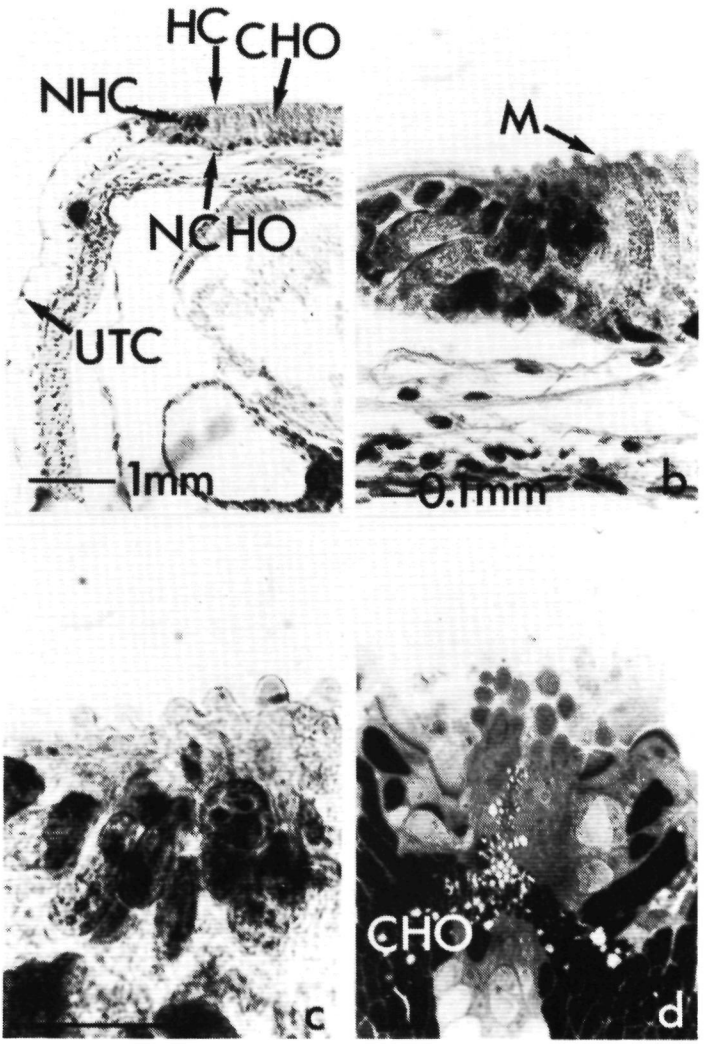

Fig. 3.2. Light microscopic representation of the hemispheric-apex cells (HC) and the cells of Hoyle's organ (CHO) in *Loligo vulgaris* and *Loligo forbesi* embryos. a: Sagittal section of the dorsal branch of Hoyle's organ in stage XX *Loligo vulgaris* embryos. The nucleus (NHC) of a hemispheric-apex cell (HC) is always situated near the central part of the cell. The cells of Hoyle's organ (CHO) have rounded nuclei (NCHO) always located near the cell base. UTC = uniform-type ciliated cell. b: HC situated at the posterior end of the dorsal branch of Hoyle's organ. M = location of microvilli. c: The long axis of the pear-shaped nucleus of the HC is parallel to the main axis of the cell. d: A semithin transversal section of Hoyle's organ and the HC immediately after hatching. The granules in the cells of Hoyle's organ (CHO) have partly disappeared due to the release of their product, *i.e.* the hatching enzyme, via multiple exocytosis. Empty spaces are evident in most CHO.

DISCUSSION

The present results show that the HC are clearly morphologically distinguishable from the CHO. The latter produce an enzyme which is released during hatching (Denucé and Formisano, 1982). Each embryo is equipped with a high amount of enzyme, sufficient to create long channels in the envelopes. This was also indicated by experiments carried out by Von Boletzky (1979a). In the present study the CHO of all juveniles fixed immediately after hatching still contained large amounts of unused hatching enzyme. As a rule most *Loligo* embryos develop near the outside of the jelly mass. At hatching they have to cross only the chorion and a thin layer of egg jelly. In this case only a small amount of enzyme is needed during hatching.

The present investigations indicate that the HC, as well as the CHO, are transient embryonic cells. In *Loligo* juveniles, the HC and CHO degenerate after hatching (Chapter 2). Recent investigations of Arnold and Singley (1989) showed that HC (α-cells) appear also in embryos of the squid *Loligo pealei*.

The function of the HC remains obscure. The location of the cells on the mantle surface and their morphological changes after hatching suggest that they may be involved in the hatching process, perhaps having a glandular role. In support of a secretory function it was noticed that after hatching the vesicles inside the HC disappear and that the content of the cells, heavily staining before hatching, acquires low electron density.

Arnold and Singley (1989) suggest that the HC are adhesive cells which attach the CHO to the inside of the chorion before hatching. Just before hatching the cell product is released as soon as the embryo stretches out its mantle tip to come into contact with the chorion. This movement is known to initiate hatching (Von Orelli, 1959).

Based on the investigations of Arnold and Singley and our results, however, also other possible functions of the HC can be proposed. First, the HC secrete their product shortly before hatching. Our own observations indicate that the hatching behaviour of *L. forbesi* and *L. vulgaris* embryos starts with strong contractions of the mantle and swimming around of the embryo while the apical part of the mantle is frequently contracted and rubs against the chorion (Chapter 8). This typical behaviour can last more than 20 minutes. It is possible that during this period a substance is released which makes the chorion suitable for digestion by the hatching enzyme and/or that a specific "hatching-spot" in the chorion is detected by the embryo. A second possibility is that the

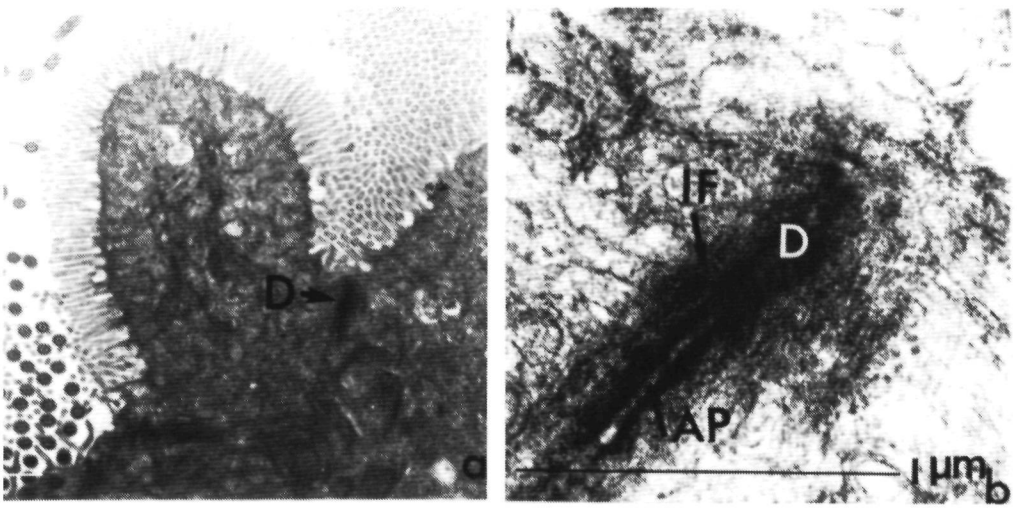

Fig. 3.3. a and b: Electron micrographs of a section of a hemispheric-apex cell showing desmosomes (D). AP = attachment plaques, IF = intermediate filament.

HC secrete their product while the embryo crosses the chorion and the layer of egg jelly. As the embryo crosses the jelly layer, the mantle tip is repeatedly stretched. Besides the fact that this way the hatchling becomes rather streamlined [according to Arnold and Singley (1989) the HC are probably capable of contraction] it is also possible that during stretching, substances are released which facilitate the functioning of the uniform-type ciliated cells and/or regulate the partial secretion of the hatching enzyme. In favour of these assumptions is also the fact that HC do not appear in *Sepia officinalis* embryos. In contrast to squid embryos, cuttlefish embryos develop in

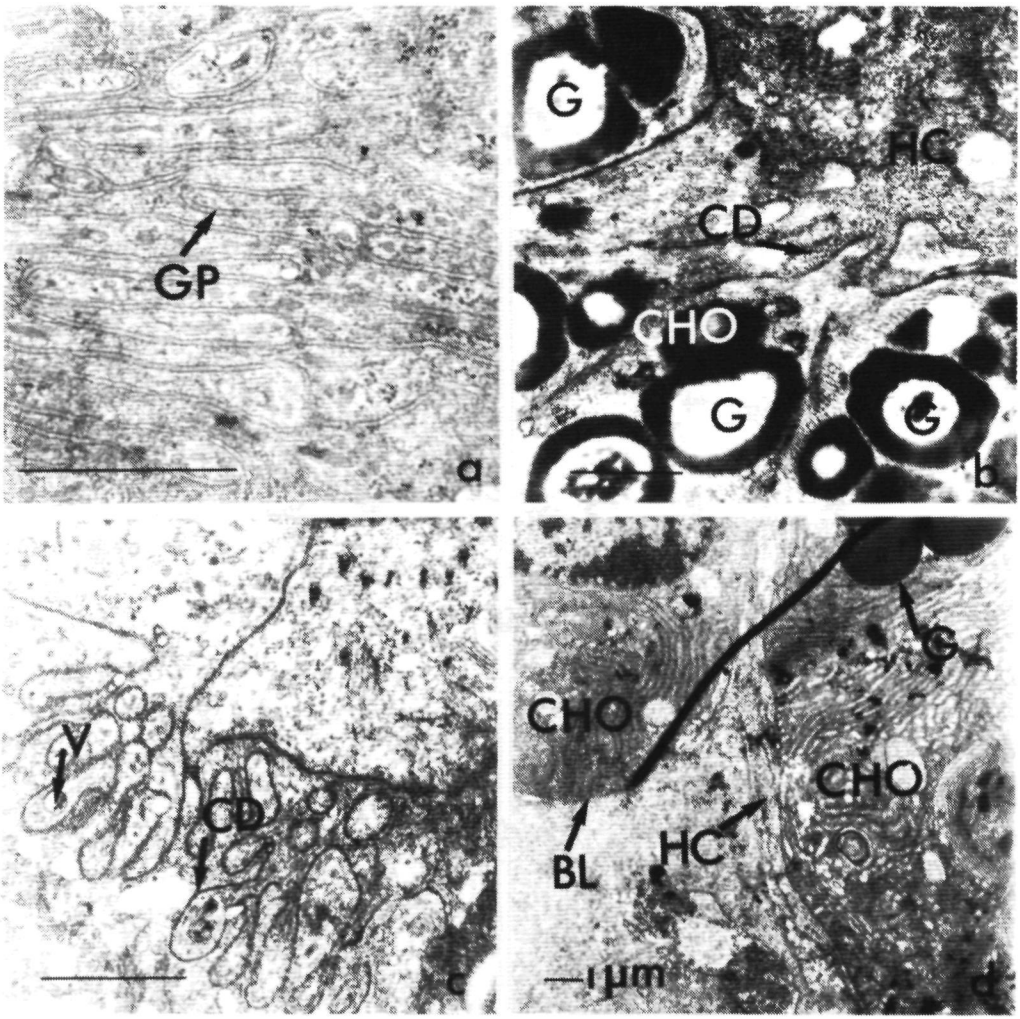

Fig. 3.4. Morphological features of the membrane of hemispheric-apex cells (HC) and the cells of Hoyle's organ (CHO). a: Cellular interdigitations of HC in stage XX *Loligo vulgaris* embryos. b: Cellular interdigitations between the HC and CHO. c: Cellular interdigitations of HC directly after hatching, showing small vesicles. d: Lower part of the HC which is characterized by a tapering end, splitting up in a T-shape, as shown in ultrathin sagittal sections. BL = basal lamina, CD = cellular interdigitations, CHO = cell of Hoyle's organ, G = granule, GP = glycoprotein layer, HC = hemispheric-apex cell, V = vesicle.

separate eggs which are, at the end of embryonic development, only covered by a thin layer of egg jelly. During hatching almost all hatching enzyme present in the CHO is released (Chapter 7).

A third possibility is that HC are absorptive cells that, during embryonic development, remove solutes from the perivitelline fluid. Recently such a transitory cell complex has been found in the gastropod *Nerita picea* [the cells are evident in trochophores but are resorbed before the veligers

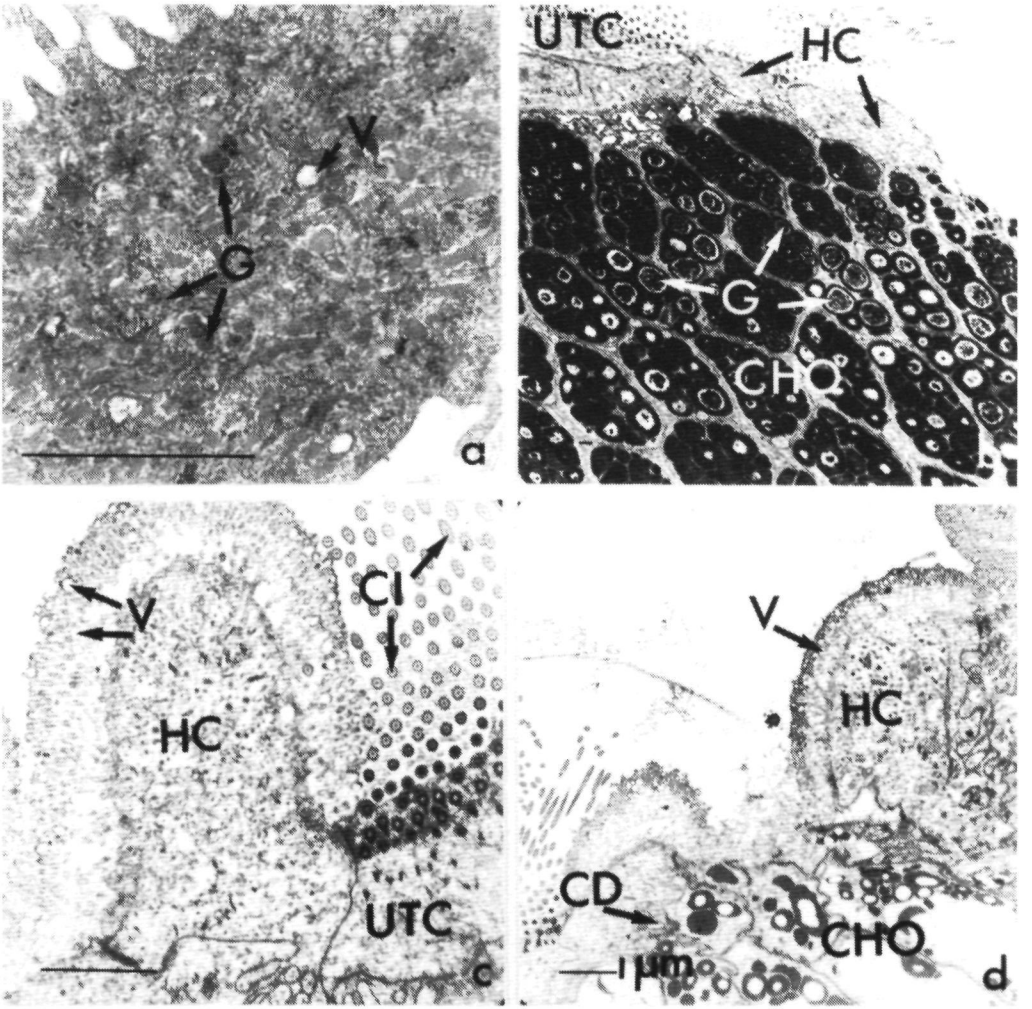

Fig. 3.5. Apical cell part of the hemispheric-apex cells (HC) and the cells of Hoyle's organ (CHO) in stage XX *Loligo vulgaris* embryos and directly after hatching. a: In stage XX many lightly staining granules and vesicles are found in the most apical part of the HC. b: Before hatching the apical part of the CHO is completely filled with rounded, electron dense secretory granules. Inside these granules electron dense and lucent regions can be discerned. c and d: The HC and the CHO directly after hatching. The interior apical part of the HC is less dense and vesicles appear between the microvilli (c). In the CHO part of the cell product is released (d). CD = cellular interdigitations, CHO = cell of Hoyle's organ, CI = cilia, G = granule, HC = hemispheric-apex cell, UTC = uniform-type ciliated cell, V = vesicle.

hatch (Rivest and Strathmann, 1989)]. These cells are also provided with microvilli and may act to absorb capsular albumen. Electron microscopic studies of epithelia with absorptive functions in vertebrates also show that the luminal surface presents many microvilli (Junqueira and Carneiro, 1980). Microvilli considerably increase the absorption capacity and the surface area of the cell, thus enhancing the efficiency of all processes occurring in this region. In the HC the absorbed substances may be released or degraded after hatching.

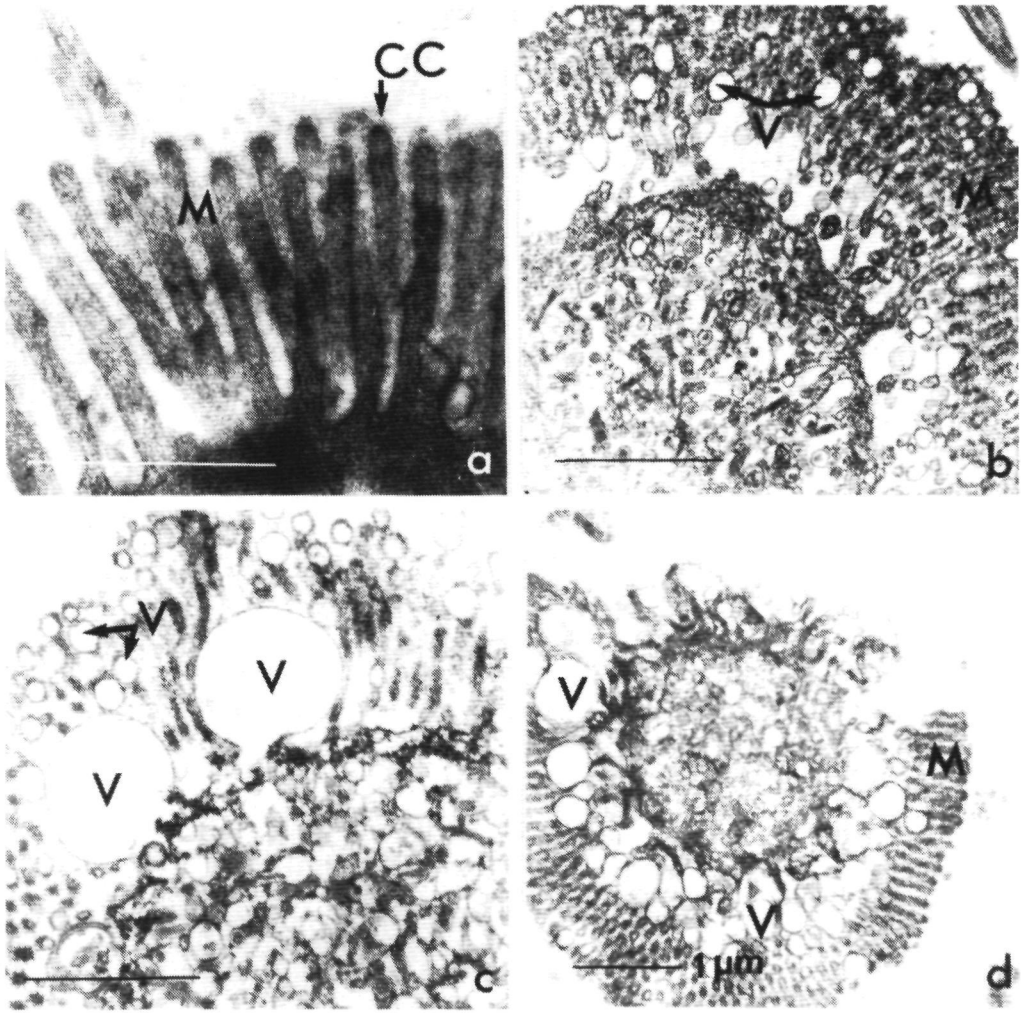

Fig. 3.6. Microvilli on the surface membrane of hemispheric-apex cells (HC) in *Loligo forbesi* at stage XX of embryonic development (a) and at different times after hatching (b to d). a: Before hatching no vesicles could be discerned. b: Directly after hatching various small vesicles can be seen. c: Two hrs after hatching. Large vesicles are present near the apical cell membrane. d: Six hrs after hatching. Many apical microvilli have disappeared. CC = cell coat, M = microvilli, V = vesicle.

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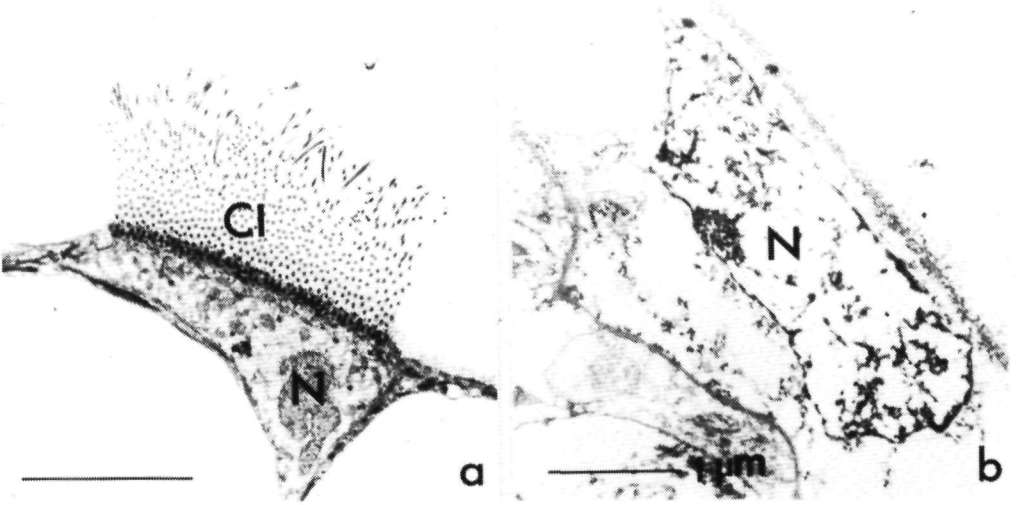

Fig. 3.7. Uniform-type ciliated cells in the epidermis of *Loligo forbesi* stage XX embryos (a) and 6 hrs after hatching (b). Cl = cilia, N = nucleus.

A STUDY OF ECTODERMAL CELL COMMUNICATION IN THE MANTLE EPITHELIUM OF *LOLIGO VULGARIS* AND *LOLIGO FORBESI* HATCHLINGS USING IONTOPHORETIC DYE INJECTION*

ABSTRACT. A total of four types of ectodermal cells in the mantle epithelium of *Loligo vulgaris* and *Loligo forbesi* hatchlings were iontophoretically injected: 1) enzyme producing cells of Hoyle's organ (CHO), 2) hemispheric-apex cells (HC), 3) uniform-type ciliated cells (UTC) and 4) mucous cells (MC). The morphology and intercellular communication were studied using the fluorescent dyes LY (Lucifer Yellow) and LY-D (Lucifer Yellow Dextran). No dye coupling was found between any type of cell when LY-D was injected. When LY was used MC and UTC showed no signs of coupling, indicating their individual nature, whereas neighbouring HC and/or CHO cells appeared to be linked. The results indicate that HC are probably activated during or just before hatching and are involved in the hatching process.

INTRODUCTION

At the end of embryonic development, the epidermis of the mantle of *Loligo vulgaris* (European squid) and *Loligo forbesi* (veined squid) (Roper et al. 1984) is differentiated into a cover of glandular cells, interspersed with ciliary cells (Arnold and Williams-Arnold, 1980; Von Boletzky, 1987a; Fioroni, 1963; Chapters 2 and 3 of this thesis). At the posterior end of the body a set of modified ectodermal cells, arranged in the form of an anchor, is found. This so-called organ of Hoyle (Hoyle, 1889) functions as the hatching gland (Jecklin, 1934; Denucé and Formisano, 1982). Situated at the intersection of the three branches of Hoyle's organ, hemispheric-apex cells (HC) are found (Arnold and Singley, 1989; Chapters 2 and 3 of this thesis). Morphologically these HC can be clearly distinguished from the cells of Hoyle's organ (CHO).

In cephalopods the embryonic phase is considered to end with the hatching process (Vayssière, 1910; Wintrebert, 1928; Hibbard, 1937; Denucé, 1984). In *L.vulgaris* and *L.forbesi* an average of 90 ovate eggs are enclosed in one and the same envelope (Jecklin, 1934; Worms, 1983). During hatching the enzyme, stored in the CHO, is released locally to dissolve the surrounding envelope with which it makes contact (Von Boletzky, 1987a). The ciliary bands of the mantle integument, which invariably beat in an anterior direction, provide most of the locomotory effort during hatching when the animal emerges from the gelatinous envelopes of the egg mass (Von Boletzky, 1979a; 1980; 1982a). The CHO as well as the ciliated cells disappear after hatching (Arnold and Williams-Arnold, 1980; Chapters 2 and 3 of this thesis).

Immediately after hatching morphological changes occur in the HC. The apical part of the cells which, before hatching, is filled with many translucent granules becomes less dense and small vesicles appear between the microvilli. The function of the HC is not known but the location of the cells on the mantle surface, in the vicinity of Hoyle's organ, the morphological changes occurring immediately after hatching and the fact that the HC undergo posthatching degeneration all

Chapter 4 is the modified version of Pauli, W P , Gevers, P , Denucé, J M (1991) A study of ectodermal cell communication in the mantle epithelium of *Loligo vulgaris* and *Loligo forbesi* hatchlings using iontophoretic dye injection Inv Rep Dev in press

suggest that they might be involved in the hatching process (Chapters 2 and 3).

If the HC are indeed related in some way to the hatching process, they probably are activated simultaneously with the CHO The CHO release their product only once, during a very short, specific period of the embryonic phase, *i e* just before and/or during hatching. Functional cell-to-cell communication may thus play some role in coordinating the activity of HC and CHO. Also the activity of the ciliated and the mucous cells could be coordinated this way but this has not been investigated thus far.

In the present study the morphology and cell-to-cell communication between ectodermal cells in the mantle epithelium of *L.vulgaris* and *L.forbesi* were investigated using iontophoretic injection of fluorescent dyes. This technique permits tracing of the overall shape of cells in detail because dye very often spreads rapidly within injected cells (Steward, 1978). High molecular weight dyes can only pass through cytoplasmic bridges. When a fluorescent dye of a relatively low molecular weight, like Lucifer Yellow (LY), is used it frequently spreads from the injected cell directly into neighbouring cells Thus cell-to-cell communication via cellular connections like gap junctions can be established (Guthrie et al., 1988; Marthy and Dale, 1989). The transfer of dye from cell to cell is termed dye-coupling (Steward, 1978).

MATERIALS AND METHODS

The study was carried out at the Catholic University in Nijmegen (Department of Zoology I), The Netherlands and at the Agricultural University in Wageningen (Department of Experimental Animal Morphology and Cell Biology), with embryos of the squids *Loligo vulgaris* and *Loligo forbesi* From January till March 1990 *L. forbesi* eggs were collected from the English Channel and were sent immediately to the Delta Institute for Hydrobiological Research (DIHO) in Yerseke (The Netherlands). *L. vulgaris* eggs were collected from the Oosterschelde (South Western part of The Netherlands) during spring 1990. At the DIHO the *Loligo* eggs were kept in an indoor aquarium (capacity 2.5 cubic litres) provided with running Oosterschelde water (salinity approximately 30 ‰, Chapters 10 and 11). To provide optimal conditions for development the egg strings were suspended vertically in the aquaria. When deposited on the bottom of the aquarium about 50% of the embryos died, especially the ones in direct contact with the bottom, probably due to lack of oxygen (Chapter 8). The eggs remained in this aquarium until the majority of the embryos in one string had reached stage XX of Naef (*i.e.* hatching stage) At this stage the external yolk sac is almost entirely absorbed (it is generally shorter than the length of arms II) and only the yolk sac envelope remains. We applied the stage descriptions of Sewaga et al. (1988), Arnold (1965a) and Naef (1923; 1928) to our material but only the stage numbering of Naef was used.

As soon as the embryos reached stage XX they were taken from the egg capsules and the chorion and were immediately anaesthetized for a period of 5 minutes using 0 8% TMS (Tricaine-methane-sulphonate, Crescent Research Chemicals, Arizona, USA) diluted in filtered Oosterschelde water (Millipore 0.5 µm). After this treatment the embryos were placed again in sea water. From each embryo one ectodermal cell was injected iontophoretically with Lucifer Yellow CH (LY; Sigma; di-Li-salt; MW 457), dissolved in 0.3 M LiCl (hyperpolarizing current, 50 nA, 5 Hz, 5 min). Glass electrodes were pulled from Clark Electromedical Instrument electrodes (GC 150F-15) with a DKI electrode puller As a control similar experiments were carried out using the relatively high molecular weight dye Lucifer Yellow Dextran (LY-D; Sigma; MW 40,000). Injections were observed using a Zeiss microscope, supplied with a 50W mercury epilight source Most injections were photographed on 400 ASA Kodak TMY 5053 film

In order to identify the injected and dye-coupled ectodermal cells, injected embryos were fixed in 4% formaldehyde in filtered Oosterschelde water, during 24 h at 4 °C in complete darkness After fixation the embryos were embedded in Technovit 7100 (Kulzer & Co., Technical Division, Bad Homburg, Germany). Lateral sections (5-7 µm) were stretched on hand-warm water and mounted on glass slides. Sections were examined under a Wild Leitz microscope (Orthoplan) LY or LY-D labeled cells were photographed Tri-X-Pan 400 ASA Kodak film.

RESULTS

Four different types of ectodermal cells in the mantle epithelium of *L. vulgaris* and *L. forbesi* embryos were injected during the various experiments: 1) enzyme producing cells of Hoyle's organ (CHO), 2) hemispheric-apex cells (HC), 3) uniform-type ciliated cells (UTC) and 4) mucous cells (MC). The results of the LY and LY-D injections are described below for each category of cells.

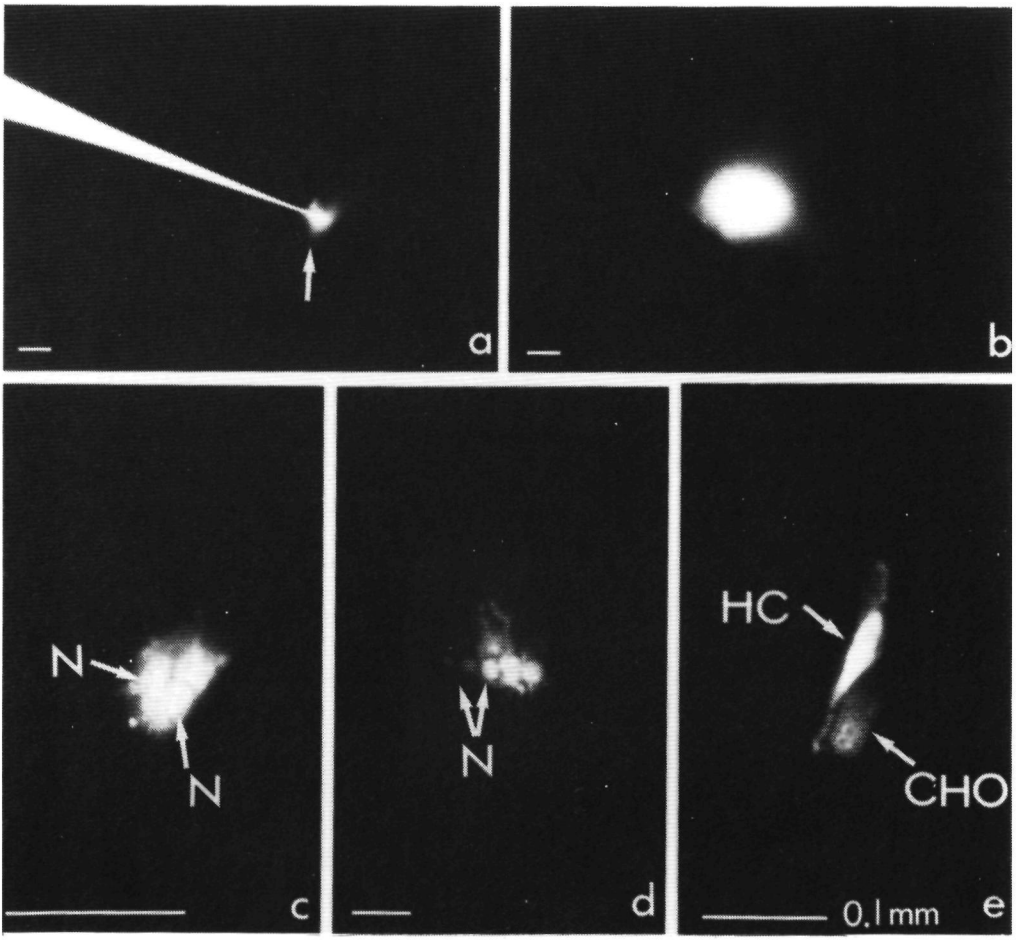

Fig. 4.1. Enzyme producing cells of Hoyle's organ (CHO). a: Already during iontophoretic injection, LY spreads from the injected CHO into neighbouring cells. Arrow: Dye-coupling is found in the basal part of the CHO. b: CHO directly after injection. LY has spread into several cells. c: A section showing two dye-coupled CHO. Fluorescence is observed in the euchromatin and in the vicinity of the nucleus. The apical part of the cells is not labeled. d: A section showing several dye-coupled CHO. LY is first observed in the nucleus and subsequently in the basal part of the cell. e: A section showing dye-coupling between a CHO and a HC. CHO: cells of Hoyle's organ, HC: hemispheric-apex cell, N: nucleus.

Hatching enzyme producing cells of Hoyle's organ

During the period of iontophoretic injection, LY began to spread from the injected CHO into neighbouring cells (Fig. 4.1a). A few seconds after the injection was completed, fluorescence was observed in at least two other CHO (Figs. 4.1b and c). After 10 minutes, LY had spread to so many cells that it was almost impossible to detect fluorescence in the sections. To avoid this, most embryos were fixed immediately after the injection was completed, whenever rapid spreading of fluorescence was observed, in order to identify the injected cell and the cells which showed dye-coupling in microscopical sections. CHO labeled with LY-D showed no dye-coupling, as the dye remained restricted to the injected cell.

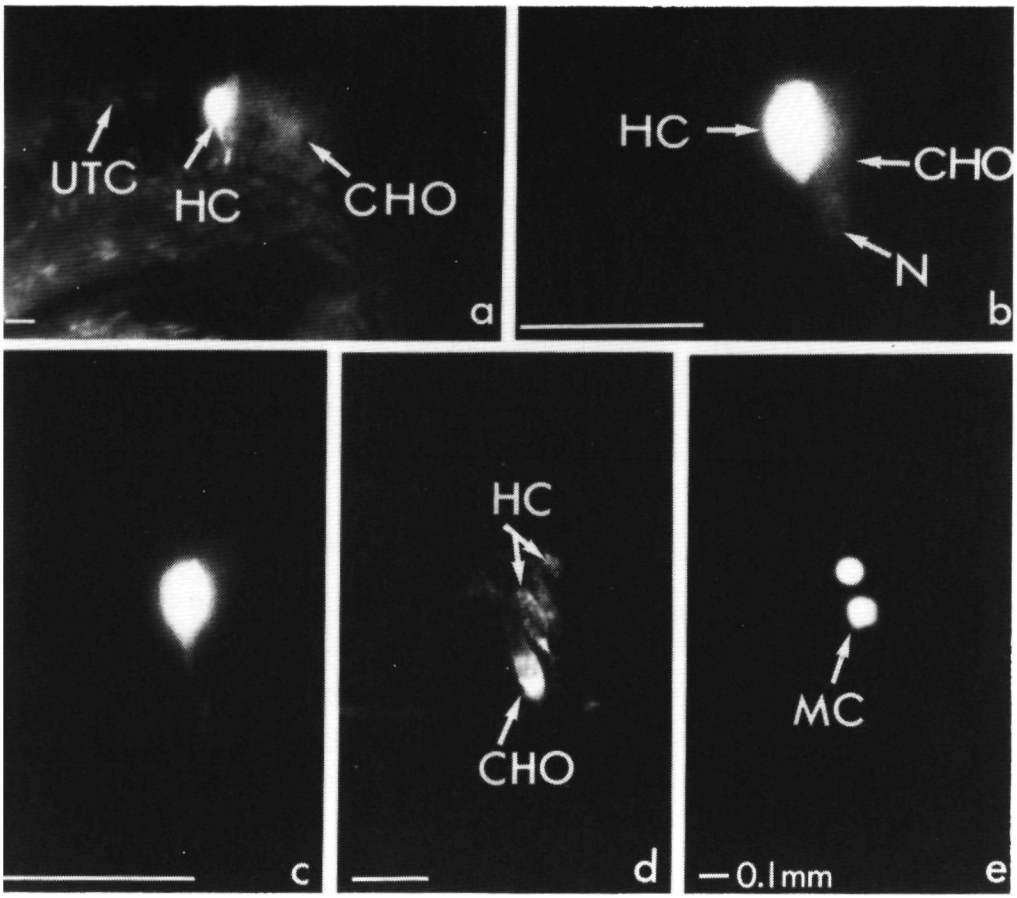

Fig. 4.2. Hemispheric-apex cells (HC). a: Section of a LY injected HC. b: Dye-coupling between a HC and a CHO. c: A section of a LY injected CH showing the middle part of the cell and the tapering distal part. d: Dye-coupling between a CHO and three HC. e: LY labeled MC a few minutes after injection showing secretion of dye. CHO: cells of Hoyle's organ, HC: hemispheric-apex cell, MC: mucous cell, N: nucleus.

In microscopical sections of LY injected CHO, most fluorescence was found in the basal part of the cell (Figs. 4.1c and d). In the same area dye-coupling was observed during injection (Fig. 4.1a). In all injected CHO fluorescence was found in the euchromatin of the nucleus and near the outside of the nucleus (Fig. 4.1c), where the Golgi complex is located (Chapter 3). In a few cases the apical part of the CHO, which contains many enzyme granules, became fluorescent, but this only occurred when a rather high amount of dye was injected into one cell. In dye-coupled CHO,

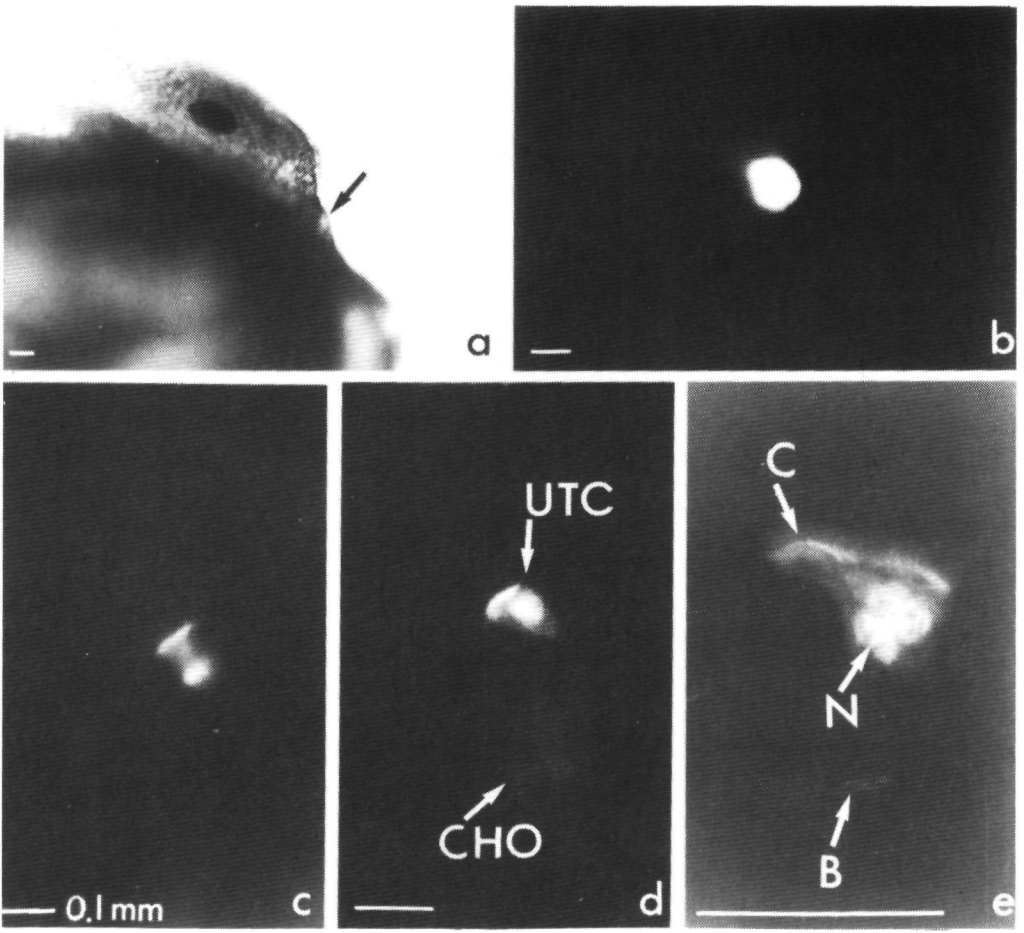

Fig. 4.3. Uniform-type ciliated cells (UTC). a: The apical part of a stage XX *Loligo vulgaris* embryo showing an injected UTC (light source: 50W mercury epilight combined with visual light). b: A UTC directly after injection. LY is found in the apical part of the cell. c: A LY labeled UTC a few minutes after injection showing fluorescence in the apical and basal part of the cell. Fluorescence is absent in the central part of the cell. d: A section of a LY-D labeled UTC in the vicinity of CHO. e: A section of a LY labeled UTC. Most fluorescence is found in the euchromatin and in the apical part of the cell, where the cilia are connected with the basal bodies. B: basal part of the UTC, C: cilia, CHO: cells of Hoyle's organ, N: nucleus, UTC: uniform-type ciliated cell.

LY was first observed in the nucleus and successively in the basal part of the cell in the vicinity of the nucleus (Fig. 4.1d)

If the neighbouring cell of an injected CHO was a HC, dye-coupling took place although the dye did not spread as rapidly as between CHO (Fig. 4.1e). Dye-coupling was never observed between CHO and UTC or between CHO and MC

Hemispheric-apex cells

Injection of the HC was not easy, probably because the apical part of HC is hemispherical and the exposed surface of the cell is rather small and covered with microvilli. Already during the iontophoretic injection, LY spread directly throughout the cell. If the embryos were fixed immediately after injection was performed the strongest fluorescence appeared in the central area of the cell, where the nucleus is located (Figs. 4.2a,b and c). The tapering proximal part of the HC, directed toward the basal lamina, showed less fluorescence but in most cases it was still recognizable (Figs. 4.2a and c). If fixation took place within a few minutes after injection, dye-coupling was found between HC and CHO and/or between HC and HC (Figs. 4.2b and d), though in the latter case dye spreading occurred more rapidly. If LY-D was injected no dye-coupling was found.

Uniform-type ciliated cells

The uniform-type ciliated cells (UTC) (Arnold and Williams-Arnold, 1980) could readily be recognized in embryos after injection. During injection of LY or LY-D the dye spread rapidly in the most apical part of the cell (Figs. 4.3a and b) A few minutes after the injection the basal part of the UTC also started to fluoresce (Fig. 4.3c), but fluorescence could not be observed in the central part of the cell. Owing to this many UTC appeared as two parallel fluorescent stripes after iontophoretic LY or LY-D injection (Fig 4.3c). LY and LY-D never spread from an injected UTC into a neighbouring cell: the dye invariably remained rather concentrated within the injected cell (Figs. 4.3d and e). Therefore and because UTC can be easily distinguished from other epidermal cells on a morphological basis, injected UTC could always be traced back in the sections without much difficulty. After fixation the highest fluorescence was found in the euchromatin of the nucleus and in the apical part of the cell (Figs. 4.3d and e).

Epidermal mucous cells

The rather large, prismatic MC of the mantle epithelium could be injected with fluorescent dyes easily. During injection of LY and LY-D the dye spread quickly throughout the cell. Some injected mucous cells, however, started to secrete the dye immediately after the injection was accomplished (Fig. 4.2e). In such cases, the secreted dye became visible as a droplet, increasing in size, just above the apical part of the injected cell. The droplet could easily be removed using a fine pipette. It was never found in microscopical sections. No dye-coupling was found between MC and other epidermal cells.

DISCUSSION

In the present study dye-coupling was never found if LY-D was injected in epidermal cells of *L. vulgaris* and *L. forbesi* hatchlings. LY-D has a rather high molecular weight (about 40,000). Of all known types of cellular contacts only cytoplasmic bridges allow passage of all molecules that can pass through gap junctions, in addition to larger molecules, such as RNA, polysomes and LY-D. Cytoplasmic bridges even allow passage of organelles such as elements of the cytoplasmic membrane system and mitochondria (Arnold, 1974). A number of studies have shown that in early cephalopod embryos, cells within a germ layer are often connected by cytoplasmic bridges (Arnold, 1974; Cartwright and Arnold, 1980, 1981; Marthy, 1982, 1985; Ginzberg et al., 1985) In particular Cartwright and Arnold (1980) presented histological evidence that small groups of early embryonic cells of common fate are connected this way. In stage XX *Loligo* embryos cytoplasmic bridges between epidermal cells were not found during the present study

During the iontophoretic injections the secretory function of MC was evident By feeding

squids neutral red solutions, this function was already observed by Kisch (1951a). The neutral red was eliminated by the skin in the form of dark red droplets. According to Kisch (1951b) the neutral red was probably combined with mucus or protein, since the droplets did not dissolve in sea water as neutral red normally does. In our experiments LY or LY-D was secreted by injected MC and the fluorescent droplets did not dissolve in the water but remained in contact with the apical part of the cells.

Marthy showed in 1974 that MC secrete a haemagglutinating substance and that the cells have a protective function: by secreting this substance, potentially harmful microorganisms and particles settling on the body surface are "captured" externally. The agglutinating substance is released even during embryonic development, which implies that MC are probably activated long before hatching. These findings are in agreement with our results in which the absence of dye-coupling between MC and other epidermal cells indicates that MC probably act individually in the epithelium and can be activated independently from other epidermal cells.

Similarly, dye-coupling was never found between UTC or between UTC and other epidermal cells. UTC are responsible for locomotion when the embryo traverses the chorion and the gelatinous envelopes of the egg mass (Von Boletzky, 1979a; 1980, 1982a). In our experience, ciliary beating of UTC can be observed in *L.vulgaris* embryos from stage XV on Arnold and Williams-Arnold (1980) suggest that during embryonic development UTC probably function, along with the ciliary tuft cells, in generating the circulation of the PVF and the movement of the older embryo. The results of Arnold and Williams-Arnold and our own observations indicate that UTC are activated before hatching and probably function independently from other epidermal cells, such as CHO and HC. This is in agreement with the present results, although it cannot be ruled out that LY became immobilized in the UTC before it was able to spread into other cells or that absence of detectable dye-coupling is due to a low permeability for the label or a low number of gap junctions. Dye transfer experiments do not permit to distinguish between these possibilities

CHO injected with LY showed spreading of label between these cells, even when fixation followed within a few minutes after injection. The observed dye-coupling may be a common property of CHO performed through cellular contacts such as gap junctions. The fact that dye-coupling was only found between CHO and between CHO and HC, and not between these cells and other epidermal cells, and the fact that the LY-D injections showed no dye-coupling, is in favour of these assumptions.

In complex, differentiated organisms intercellular communication is often necessary to coordinate the activities of individual cells (*e g.* Pitts and Finbow, 1986) Various types of cellular connections, like gap junctions, provide possible routes for chemical communication In cephalopod embryos gap junctions have only been investigated in very early developmental stages (Ginzberg et al 1985, Marthy and Dale, 1989). The CHO as well as the HC are transient embryonic cells which degenerate after hatching (Chapters 2 and 3) CHO release their product, the hatching enzyme, during emergence, which is a rather short, specific period in the embryonic phase (Chapter 3). Because hatching enzyme is used only once and has to be released at a specific moment, cellular contact sites, like gap junctions, probably coordinate the activities of CHO. In this way, all CHO can be activated at once while only a few cells, or even one cell, need to be stimulated Our results also demonstrated dye-coupling between individual HC and between HC and CHO. These results favour the idea that the HC are probably activated during or just before hatching (Arnold and Singley, 1989, Chapter 3 of this thesis) and are involved in the hatching process.

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CYTOCHEMICAL ANALYSIS OF LECTIN LABELING IN MANTLE EPITHELIAL CELLS OF *LOLIGO VULGARIS*, *LOLIGO FORBESI* AND *SEPIA OFFICINALIS* EMBRYOS AND JUVENILES*

ABSTRACT. This paper describes the results of a histochemical study in which fluorescein isothiocyanate (FITC) labeled lectins served as reagents to detect and characterize carbohydrate containing macromolecules in mantle epithelial cells of *Sepia officinalis*, *Loligo vulgaris* and *Loligo forbesi* embryos and juveniles. It appears that the application of FITC labeled lectins is an effective method for characterizing cells and organs in decapod cephalopod embryos. In the mantle epithelium of all species examined, the cells of Hoyle's organ (CHO) could very readily be distinghuished after using *Ulex europaeus* (UE I) lectin Because the UE I binding sites are probably exclusively restricted to CHO granules our investigations provide circumstantial evidence that the hatching enzyme of *L.vulgaris*, *L.forbesi* and *S.officinalis* is a α-L-fucose containing glycoprotein. The most suitable lectin to label the hemispheric-apex cells (HC) appeared to be *Canavalia ensiformis* lectin (Con A) because it did not stain any other cell type in the mantle epithelium. The Con A binding sites were found in the cell coat and in the supranuclear area, probably inside the granules. Most likely the positive reaction can be ascribed to glucose or mannose residues It is concluded that HC and CHO cannot only be distinguished with morphological criteria but also by their specific carbohydrate characteristics.

INTRODUCTION

In the embryonic mantle epithelium of the two squid species *Loligo forbesi* and *Loligo vulgaris*, and the cuttlefish *Sepia officinalis* three different ectodermal cell types are found at the end of embryonic development: (1) the uniform-type ciliated cells (UTC) which provide the locomotory effort during hatching (Arnold and Williams-Arnold, 1980, Von Boletzky, 1979a; 1980; 1982a), (2) the mucus secreting cells (MC) (Kisch, 1951a; 1951b) and (3) the enzyme producing cells of the gland of Hoyle (CHO) which functions as the hatching gland (Hoyle, 1889; Jecklin, 1934; Denucé and Formisano, 1982). In the loliginid species *Loligo pealei* (Arnold and Singley, 1989), *L.vulgaris* and *L.forbesi* (Chapters 2,3 and 4) a fourth cell type is found. These so-called hemispheric-apex cells (HC) are situated at the intersection of the three branches of Hoyle's organ The function of the HC is still unknown but there is circumstantial evidence that the HC are involved in the hatching process (Arnold and Singley, 1989; Chapters 2, 3 and 4 of this thesis).

To investigate the function of the HC we recently applied some classical histochemical staining methods. During these experiments it became evident that the UTC, MC, CHO and HC are PAS-positive cells. The periodic-acid-Schiff (PAS) procedure, although widely used, in fact does

* Chapter 5 is the modified version of Paulij W P , Verspaandonk, C J M , Verberne, M G A H , Denucé, J M (1991) Cytochemical analysis of lectin labeling in mantle epithelial cells in *Loligo vulgaris*, *Loligo forbesi* and *Sepia officinalis* embryos and juveniles Inv Rep Dev in press

not indicate more than the generic presence of vicinal glycol groups (Faraggiana et al., 1982) In more recent years lectins have been used with increasing frequency as histochemical markers for various sugars (Etzler and Branstrator, 1974; Katsuyama and Spicer, 1978). In their interaction with saccharides, lectins serve as models for carbohydrate-specific antibodies, with the important advantage that it is possible to purify lectins in gram quantities (Goldstein and Hayes, 1978). The specificity of lectins is proved to be much more exquisite than originally assumed, since they not only distinguish between different monosaccharides, but also specifically bind to oligosaccharides, detecting subtle differences in complex carbohydrate structures (Sharon and Lis, 1989). In the present chapter we report the results of a histochemical study in which fluorescein isothiocyanate (FITC) labeled lectins served as reagents to detect and characterize carbohydrate-containing macromolecules in mantle epithelial cells of *S.officinalis*, *L.vulgaris* and *L.forbesi* embryos and juveniles.

MATERIALS AND METHODS

Biological Material

Spawns of *Loligo vulgaris* and *Sepia officinalis* attached to fishing nets in the Oosterschelde (South Western part of The Netherlands) were collected by fishermen during spring and summer of 1989 and 1990. *Loligo forbesi* spawns were obtained from the English Channel during May and June of 1989 and were sent in very early developmental stages to the Delta Institute for Hydrobiological Research at Yerseke (The Netherlands). The *Loligo* spawns were separated into individual strings and suspended vertically in an indoor aquarium (capacity 15 litres). Individual *Sepia* eggs were laid on the bottom of a perforated plastic box, suspended in an aquarium (capacity 50 litres). All aquaria were provided with running Oosterschelde water.

During embryonic development the condition and the external morphological features of the embryos were checked. To stage the *Loligo* embryos we applied the stage descriptions of Sewaga et al. (1988), Arnold (1965a) and Naef (1923, 1928) but only the numbering of Naef was used (stage XX = hatching stage). For *Sepia* we used the stage descriptions and numbering of Lemaire (1970) (stage 30 = hatching stage). Embryos were collected starting from stage X (*Loligo*) or 20 (*Sepia*). As soon as the *Loligo* embryos reached stage XX, several strings were removed from the aquarium and placed for two minutes in complete darkness. During this dark-shock most embryos hatched (Chapter 8). After darkness the egg strings were removed immediately and juveniles were fixed at intervals of 5, 10, 15, 30, 60, 90, 360 and 720 minutes after hatching. Juveniles of *Sepia* were collected every morning from the aquarium. These embryos had hatched the night before and were not older than 10 hrs at the time of collection.

Fixation

Embryos and juveniles were fixed in Hollande's fixative (Gurr, 1962), dehydrated and embedded in paraffin according to conventional histological procedures. For light microscopy, sections (5 µm) were stained with haemalum-eosin (HE)

Lectin Incubations

Sections (5 µm) were treated according to Faraggiana et al. (1982), with minor changes. Briefly, deparaffinized sections were rinsed in 0 05 M phosphate buffered saline (PBS), pH 7.2, containing 0.1 M NaCl. The sections were then incubated overnight with fluorescein isothiocyanate (FITC) labeled lectins (Sigma Chemical Co., USA), diluted in the same buffer, at 4°C in complete darkness Finally, the sections were rinsed in PBS and embedded in glycerol. The optimal concentration and the sugar specificity for each lectin are reported in Table I As controls each lectin solution was used after the addition of the appropriate inhibiting sugar at a concentration of 0.2 M (Con A and LO : D (+) mannose, HP: N-acetyl-D-galactosamine, TV : N-acetyl-D-galactosamine, UE I : L (-) fucose), except for the lectins PHA-P, PHA-L and PHA-E. The sections were examined under a Wild Leitz microscope (Orthoplan), supplied with a 50W mercury epilight source, and photographed on Tri-X-Pan 400 ASA Kodak film.

Table 5.I. Specificity and optimal concentrations of lectins.

Lectin	Source	Optimal concentration (mg/ml)	Carbohydrate Specificity
Con A	*Canavalia ensiformis*	0.05	d (+) mannose> d (+) glucose
HP	*Helix pomatia*	0.1	N-acetyl-D-galactosamine> N-acetyl-D-glucosamine
LO	*Lathyrus odoratus*	0.2	d (+) mannose> d (+) glucose> N-acetyl-D-glucosamine
PHA-E	*Phaseolus vulgaris*	0.1	oligosaccharide
PHA-L	*Phaseolus vulgaris*	0.1	oligosaccharide
PHA-P	*Phaseolus vulgaris*	0.1	oligosaccharide
TV	*Triticum vulgare*	0.05	N-acetyl-D-glucosamine> sialic acid
UE I	*Ulex europaeus*	0.1	L (-) fucose

RESULTS

Lectin labeling in *L.vulgaris* and *L.forbesi* embryos

The results of the various FITC labeled lectin incubations using stage XX *L.vulgaris* and *L.forbesi* embryos are given in Fig 5.1. UE I lectin labeled very specifically the CHO (Fig. 5.1A). Binding of this lectin was limited to the supranuclear part of the cells. The observed fluorescence was speckled, resembling strikingly the structure of the many granules which are always found in the supranuclear part of the CHO (Chapter 3). Fluorescence was also observed in the central part of the MC.

TV lectin showed reactivity in the apical part of the CHO and the HC, directly above the nucleus, and also in the apical part of the UTC (Fig 5 1B). In the CHO the observed fluorescence was mostly weak compared to the reaction found in the HC and UTC. TV lectin was not evenly distributed in the apical part of the CHO but many small unlabeled spots were detected. In this case the

unlabeled spots resembled the structure of the granules. Besides in the epidermal cells, fluorescence was also observed in the internal yolk sac, the gills and in the epithelium of the funnel gland.

The most specific labeling for the HC was obtained with the lectin Con A (Fig. 5.1C). Con A labeled the supranuclear region of the HC. In stage XX *Loligo* embryos many translucent granules are found in this part of the HC (Chapter 3). The tips of the microvilli, which appear on the extruding parts of the HC, were only weakly stained, whereas the rest of the microvilli remained unlabeled and could therefore very easily be distinguished from the apical membrane. Con A binding sites were also observed in the basal lamina, the apical parts of the secreting cells of the funnel gland,

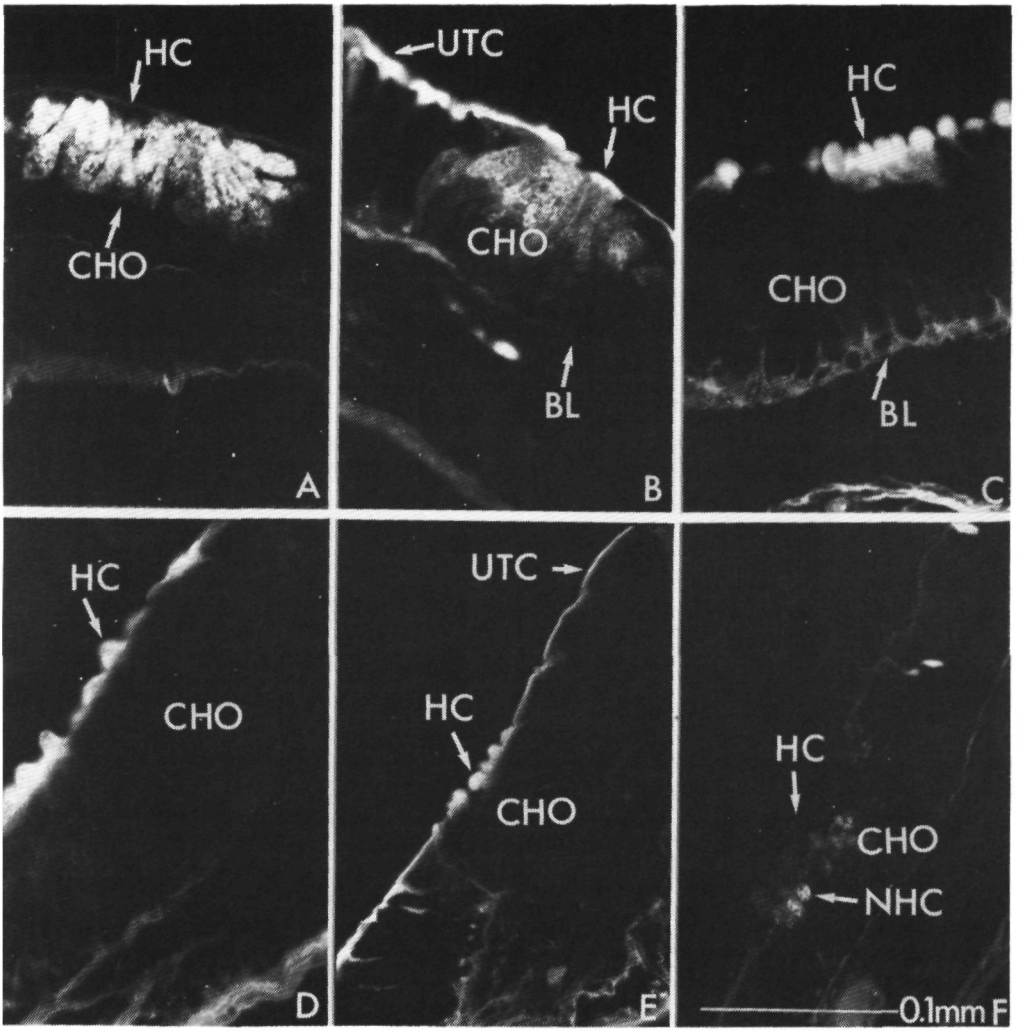

Fig. 5.1. Labeling pattern of various FITC labeled lectins in epidermal cells of the mantle epithelium of *Loligo forbesi* embryos. A) *UEI*, B) *TV*, C) *Con A*, D) *LO*, E) *HP* and F) *PHA-E* lectin (see also Table 5:I). BL: basal lamina, CHO: cells of Hoyle's organ, HC: hemispheric-apex cells, NHC: nucleus of hemispheric-apex cell, UTC: uniform-type ciliated cell.

the internal yolk sac and in the retina. Con A labeling was never observed in the CHO or the UTC.

LO lectin was used in two different concentrations. At 0.2 mg/ml the very apical parts of the HC were labeled (Fig. 5.1D). Using lower concentrations the fluorescence in the HC decreased. At a concentration of 0.1 mg/ml lectin binding was still found in the vascular system, the gills, a few epidermal cells of the tentacles, the luminal part of the cells of the ink gland and very faintly in the secreting cells of the funnel gland.

The HP lectin labeled the apical parts of all epidermal mantle cells, including the tips of the microvilli of the HC (Fig. 5.1E). The CHO remained unstained. Labeling was also found in the internal yolk sac, the funnel gland, the ink sac and the posterior salivary glands.

With the lectins PHA-L and PHA-P no reaction was found in the mantle epithelial cells. With PHA-E fluorescence was observed in the nuclei of the HC and in some epidermal cells of the tentacles (Fig 5.1F). The latter were also labeled with PHA-L at a concentration of 0.2 mg/ml.

No differences were found in lectin reactivity between *L.vulgaris* and *L.forbesi*. If the lectins were incubated with the appropriate inhibiting sugar the described fluorescence was never observed.

Lectin labeling in epidermal cells of *Sepia officinalis*

The same lectins used in the experiments with *L.forbesi* and *L.vulgaris*, with the exception of PHA-E and TV, were also applied to *S.officinalis*, but only the cells of the epidermis were studied. Like in *Loligo*, positive labeling of the granules of the CHO was found after using UE I (Fig. 5.2). This lectin also stained the MC. A very vague staining in the CHO was found after incubation with LO.

Con A and LO labeled the apical parts of the ciliated cells and the MC, close to the cell surface. Binding sites for the HP lectin were found in the central region of the MC and also on the outer surface of all epidermal cells. The cilia of the ciliated ectodermal cells and most cell membranes were labeled with PHA-P. PHA-L reactivity was found between the cilia of the ciliated cells, near the surface of all epidermal cells and in most ectodermal cell membranes.

UE I labeling before and after hatching

As UE I lectin very specifically labeled the granules in the apical part of the CHO in the three cephalopod species, this lectin was applied throughout embryonic development to visualize the development of Hoyle's organ. In all instances where UE I labeling was observed, we also found granules in the apical and middle part of the CHO in corresponding HE stained slides.

In embryos of *L.vulgaris* and *L.forbesi* lectin binding was observed in the developing CHO from stage XII on (Fig. 5.3A). In stage XII the fluorescence was still weak but the amount and the intensity

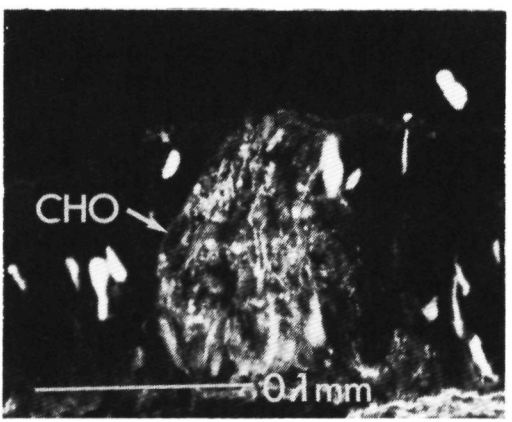

Fig. 5.2. Labeling of UE I lectin in cells of Hoyle's organ (CHO) of *Sepia officinalis* embryos.

of labeling increased very fast during embryonic development. Already in stage XIV the granular structure of the labeling became visible (Fig. 5.3B). From stage XV on the organ of Hoyle developed very rapidly (Fig. 5.3C). The CHO increased enormously in size and already in stage XVIII (Fig. 5.3D) the intensity of labeling was almost as high as in stage XX, where it reached the highest peak (Fig. 5.3E). In stage XX CHO were completely stacked with UE I binding sites, except for the basal part of the cells.

Directly after hatching it appeared that in most CHO the amount of UE I labeling had decreased (Fig. 5.3F). However, most cells still contained UE I binding sites. On these sites the fluorescence intensity had not changed. A declining fluorescence intensity was observed between 10 and 60 minutes (Figs. 5.4A and B) after hatching. Beyond this period the labeling was very weak and in

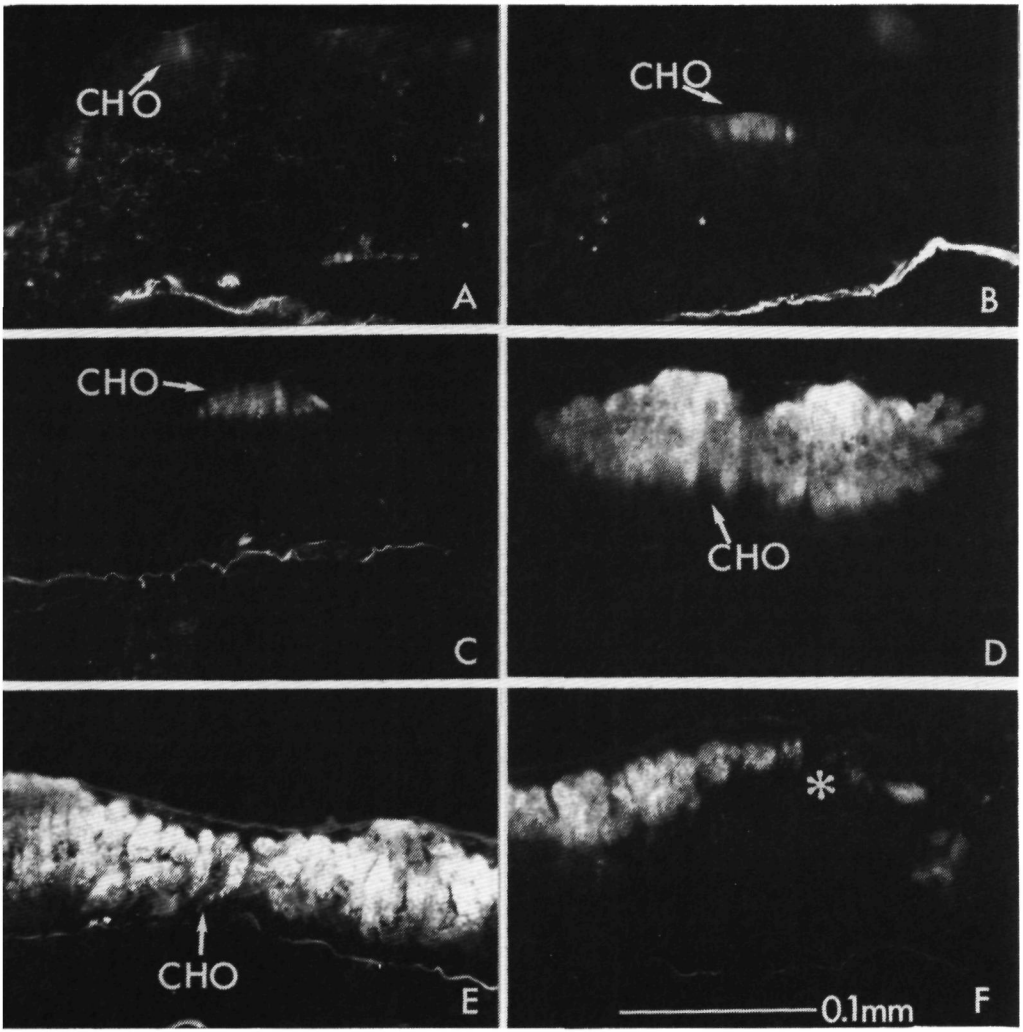

Fig. 5.3. UE I labeling in cells of Hoyle's organ (CHO) of *Loligo forbesi* during embryonic development and directly after hatching. A: stage XII, B: stage IV, C: stage XV, D: stage XVIII, E: stage XX and F: directly after hatching. *=release of UE I binding sites.

many cases fluorescence was lacking entirely. As a result of the declining fluorescence intensity the granular structure of the labeling became more pronounced.

Also in *Sepia* UE I lectin was a useful label to describe the changes in the CHO during development and hatching. UE I binding sites were first found in stage 24, where the reaction was very faint and almost uniformly localized in the CHO, except in the very basal part. The intensity and quantity of labeling increased in the course of development. Also the localization of UE I reactivity in the CHO shifted: in many instances labeling in stage 30 embryos was restricted to the very apical part of the cells. In *Sepia* juveniles UE I labeling was mostly absent.

Con A labeling before and after hatching

Con A was used to visualize the development of the HC in embryos of *L.vulgaris* and *L.forbesi*. HC are absent in embryos of *S.officinalis* (see Chapter 2). In stage XIV of *Loligo* fluorescence first appeared in the central region of the HC while the apical part of the cells remained unlabeled (Fig. 5.5A). From stage XV on the HC started to extrude above the surface of the mantle epithelium. Labeling was also found in the extruding part of the cells (Fig. 5.5B). In stage XX the quantity of labeling was maximal. At the end of embryonic development Con A binding was limited to the middle and apical region of the HC, above the nucleus. Very little labeling appeared near the tips of the microvilli (Fig. 5.5C).

Immediately after hatching Con A reactivity was observed between the microvilli (Fig. 5.5D). It increased between 5 and 720 minutes after hatching (Figs. 5.5E and F). In the middle and apical parts of the HC the fluorescence decreased. Occasionally already 60 minutes after hatching fluorescence was completely absent in the apical and middle parts of the HC, while a high amount of labeling could still be seen between the microvilli (Fig. 5.5F).

DISCUSSION

Our results indicate that the application of FITC labeled lectins is an effective method for characterizing cells and organs in decapod cephalopod embryos. In the mantle epithelium of *L.vulgaris*, *L.forbesi* and *S.officinalis*, CHO could very easily be distinghuished after using UE I lectin. We also found that UE I binding sites in the mantle epithelium are exclusively restricted to CHO granules. According to Yung Ko Ching (1930) the CHO of *L.vulgaris* start to differentiate in stage X but the first small granules can only be detected in stage XII. In our experiments UE I binding sites in CHO of *Loligo* were found from stage XII onwards, in the supranuclear region, resembling exactly the structure and location of the hatching enzyme granules. In *Sepia* we obtained similar results. In CHO of the cuttlefish the first granules are found in stage XII-XIII of Naef (Yung Ko Ching, 1930), which corresponds to stage 24-25 of Lemaire (1970). In the same embryonic stage speckled UE I labeling appeared.

In both *Sepia* and *Loligo* embryos the amount of UE I labeling augmented during embryonic

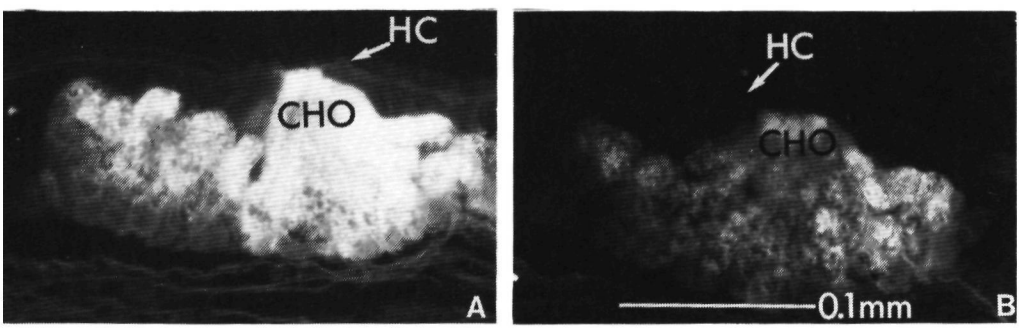

Fig. 5.4. UE I labeling in cells of Hoyle's organ (CHO) of *Loligo forbesi* juveniles 10 minutes (A) and 60 minutes (B) after hatching. HC: hemispheric-apex cells.

development, while the location of the labeling in the cells remained exactly the same. Directly after hatching the amount of labeling in juveniles decreased but in *Loligo* UE I binding sites could still be detected. These results are in agreement with investigations by Von Boletzky (1979a)(*cf.* Chapter 3 of this thesis). According to Von Boletzky (1979a) *Loligo* embryos are equipped with a high amount of hatching enzyme, sufficient to create long channels inside the envelopes, but in most cases only a small amount of enzyme is needed during hatching. TEM investigations of the mantle epithelium of *L.vulgaris* and *L.forbesi* indicated that directly after hatching CHO still contain large amounts of granules (Chapter 3).

CHO are transient embryonic cells which degenerate after hatching (Arnold and Williams-Arnold, 1980; Arnold and Singley, 1989; Chapters 2 and 3 of this thesis). Our recent results indicate that the intensity of labeling in the "unused" granules inside degenerating CHO declined after hatching. The declining fluorescence can be explained from extracellular breakdown after hatching.

In CHO of *Sepia* embryos the labeling pattern of UE I was not as compact as in *Loligo*. In *Sepia* juveniles UE I labeling was mostly absent. These results are in agreement with the investigations of Von Boletzky (1979a) and Matsuno and Ouji (1988). Sepioids enclose individual eggs in gelatinous envelopes which become thinner during embryonic development as a result of stretching (*cf.* Lemaire, 1971). During hatching the embryos only have to cross a very thin barrier so that only a small amount of enzyme is needed. Ultrastructural studies of Matsuno and Ouji (1988) on the hatching organ of *Sepiella japonica* indicated that only a few granules of the CHO are discharged during embryonic development. Most of the material is retained up to the stage just before hatching, to be discharged all at once.

UE I is a L-fucose binding lectin: inhibition analysis of purified UE I lectin has revealed a binding site complementary to α-L-fucosides (Goldstein and Hayes, 1978). According to Menghi et al. (1989b) reactivity of UE I is due to the presence of α-L-fucose in a terminal position and furthermore UE I seems to recognize fucose bound to galactose (Menghi et al., 1989a). Because UE I labeling is most likely restricted to the granular contents of CHO, our investigations provide circumstantial

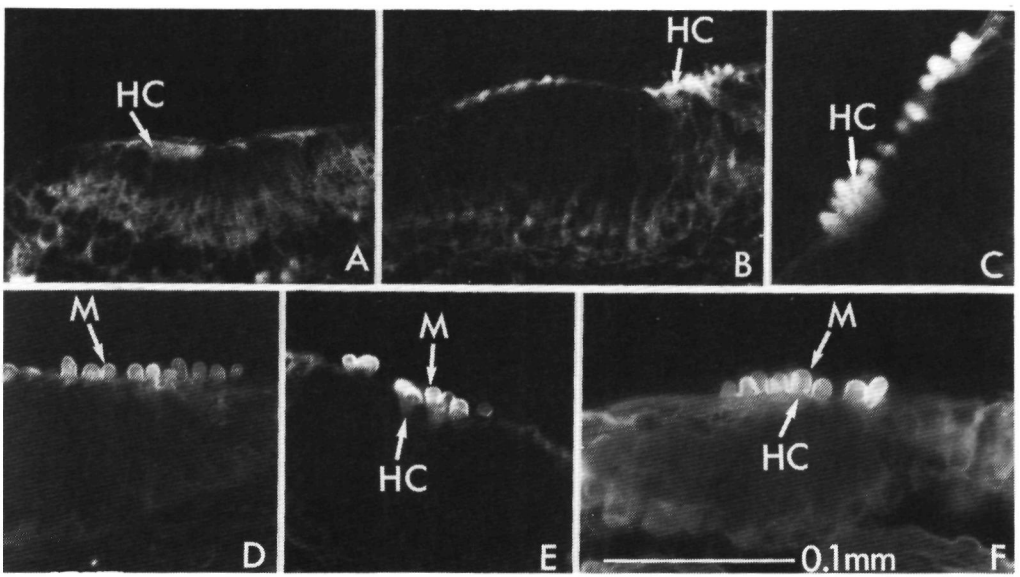

Fig. 5.5. Con A labeling in hemispheric-apex cells (HC) of *Loligo vulgaris* during embryonic development and after hatching. A: stage XIV, B: stage XV, C: stage XX, D: almost directly after hatching, E: 30 minutes after hatching and F: 60 minutes after hatching. M: microvilli.

evidence that the hatching enzyme of *L.vulgaris*, *L.forbesi* and *S.officinalis* is a α-L-fucose containing glycoprotein. Glycoproteins functioning as a hatching enzyme have been described in embryos of fish and sea urchins. In 1989 Lepage and Gache demonstrated the glycoprotein character of the hatching enzyme of the sea urchin *Paracentrotus lividus*. Experiments of Schoots and Denucé (1981) showed that the hatching enzyme of the pike *Esox lucius* is a glycoprotein containing 2% carbohydrate.

If TV lectin was used a weak reaction was observed in the apical region of the CHO. However, these TV binding sites apparently were not limited to the granules, because after incubation with the lectin many unlabeled spots in the apical part of the CHO resembled the structure of the granules.

The present study indicates that the most suitable lectin to label HC is Con A because no other cells in the mantle epithelium were stained. Besides the supranuclear part of HC, Con A also labeled the microvillar tips before hatching. According to previous TEM investigations these microvilli are covered by a cell coat (Chapter 3). A cell coat is frequently an extracellular glycoprotein layer (Junqueira and Carneiro, 1980). Because Con A reactivity is generally considered to be due to mannose, glucose and N-acetyl-D-glucosamine (Goldstein and Hayes, 1978), the cell coat of the HC probably exhibits one or more of these carbohydrate residues.

Our recent results are in favour of the assumption that Con A binding sites are found inside the granules of HC. Using Con A the intracellular location of the labeling coincided entirely with the location of the translucent granules in these cells during embryonic development and after hatching (Chapter 3). In cells which produce glycoproteins, like the goblet cell of the intestinal mucosa, the complex, high molecular weight carbohydrates are synthesized both in the endoplasmic reticulum and the Golgi apparatus. The cell product is then stored in weakly staining granules which gradually fill the apical pole of the cell (Junqueira and Carneiro, 1980). In HC of stage XIV embryos, Con A labeling was found in the central part of the cell, where the Golgi apparatus is located. From stage XV onwards labeling was also found in the extruding part of the cells. In stage XX the labeling appears maximal in the middle and extruding part of HC, where also many weakly staining granules are found (Chapter 3).

After hatching an increasing amount of Con A labeling was observed between the microvilli while the fluorescence in the extruding and middle part of the HC decreased. Previous TEM investigations indicated that after hatching the apical part of the HC becomes less dense while small, lucent vesicles, increasing in size, appear between the degenerating microvilli (Chapter 3). This explains the Con A labeling pattern after hatching. The vesicles which contain carbohydrate residues with Con A binding sites, release their product during or directly after hatching.

The Con A labeling of the retina of *L.vulgaris* and *L.forbesi* embryos is in agreement with the findings of Taba et al. (1989) who described and characterized lectin binding sites in the cephalopod retina. These Con A binding sites are probably restricted to mannose residues.

The HC were not only labeled by Con A lectin but fluorescence was also observed after the lectins TV, LO or HP were applied. With the latter three lectins labeling was only found in the very apical part of the cells where it was probably not restricted to the vesicle content.

Although we concentrated on the cells of the mantle epithelium our investigations also indicated that the secreting cells of the funnel gland were stained with Con A, TV, LO and HP. The function of this merocrine gland is unknown (Fioroni, 1978).

On the basis of the results presented, it is difficult to identify the carbohydrates at the reactive sites. Most likely the positive reaction of TV, LO, Con A and HP lectin can be ascribed to glucose, mannose and/or galactose residues. In some cases, like TV lectin, labeling can also be ascribed to sialic acid but in cephalopods this substance has only been detected in the digestive gland. It is not clear whether sialic acid is a steady component of this gland, or has been ingested with the food (Hunt, 1970).

From the present study it can be concluded that in addition to morphological criteria (Arnold and Singley, 1989; Chapters 2, 3 and 4 of this thesis), a biochemical distinction between HC and CHO is possible on the basis of the specific carbohydrate characteristics of these cells after using Con A and UE I labeling.

ACKNOWLEDGEMENTS

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PARTIAL PURIFICATION AND CHARACTERIZATION OF *LOLIGO VULGARIS* HATCHING ENZYME OBTAINED FROM HATCHING MEDIUM*

ABSTRACT. Hatching medium of *Loligo vulgaris* contains a protease which is absent in perivitelline fluid (PVF) during embryonic development and in sea water. As the enzyme is released by the embryos right before and during hatching, it can be defined as the hatching enzyme. The protease has a pH optimum of 8.5 and is probably a metalloprotease. The proteolytic activity was completely lost after three times freezing and thawing as well as after storage at -20°C for more than four months Purification of *L.vulgaris* hatching enzyme from hatching medium was hampered because of the presence of a haemagglutinin in the PVF and the scarcity of crude hatching medium.

INTRODUCTION

Despite the fact that in cephalopods different hatching mechanisms exist, hatching is always brought about by forces or agents of embryonic origin.

First, tearing up of the egg covering may be (partially) caused by mechanical forces such as muscular contractions, an increase in volume of the embryo, or puncturing by specialized embryonic structures (Schoots, 1982) In cephalopods the latter mechanism acts in embryos of *Sepiola robusta* and *Rossia macrosoma* which have a tough spine-like organ (terminal spine) on the mantle, associated with the hatching gland (Von Boletzky, 1982a). In these sepiolids the outer coat of the egg becomes very rigid a few hours after spawning and will remain so throughout embryonic development (Roper et al , 1984). At hatching the egg capsule is mechanically forced open by the terminal spine which forms the most advanced structure when the mantle edge penetrates the hatch opening (Von Boletzky, 1987a).

Second, an increase in volume of the perivitelline fluid (PVF) due to osmotically active compounds may be important during hatching. An example of (partially) osmotic hatching in cephalopods is found in *Nautilus*. Up to the present very little is known of the hatching equipment of *Nautilus*, but it is conceivable that the outermost coat of the egg unfolds under the pressure of the expanding chorion and the inner coat, at least partially exposing the latter to the environment (Arnold and Carlson, 1986; Von Boletzky, 1987a) Also, in many decapods the chorion expands under the osmotic pressure of the PVF, which greatly facilitates hatching (De Leersnyder and Lemaire, 1972; Lemaire, 1971).

Third, there is the possibility of lysis of the envelope by a hatching enzyme. In many cephalopod species the proteolytic enzyme produced in Hoyle's organ is responsible for the enzymatic degradation of the egg capsule during hatching (Denucé and Formisano, 1982).

In most cephalopod species rupture of the protective layers around the egg is accomplished by a combined action of several mechanisms. For example, the elongated eggs of *Octopus vulgaris* are surrounded only by the chorion which tightly encloses the embryo (Mangold, 1983). During embryonic development the chorion slightly expands, due to the enlargement of the embryo, from an initial size of 2.2 x 0.95 mm to 2.75 x 1.1 mm at hatching Like nearly all incirrate

Chapter 6 is the modified version of Pauly W P , Verhoof, H C C M , Denuce, J M (in preparation) Partial purification and characterization of *Loligo vulgaris* hatching enzyme obtained from hatching medium

octopods, *O.vulgaris* has a peculiar integumental organ, consisting of sets of pits with bundles of organic "setae" known as Kolliker's tufts (Fioroni, 1978). The development of this organ was studied by Fioroni (1962), its fine structure and function by Von Boletzky (1966; 1969, 1973). In *O.vulgaris* hatching begins when the apical pole of the chorion is dissolved by the enzyme released from the ruptured cells of Hoyle's organ. The latter consists, not only in *O.vulgaris* but in all incirrate octopods, of a simple transverse band of glandular cells (Fioroni, 1978). The Kolliker bundles help the hatchlings to liberate themselves from the still tightly fitting chorion (Von Boletzky, 1982a)

The hatching mechanism in *Loligo vulgaris* is based on the coordinated action of the hatching enzyme (Denucé and Formisano, 1982), which locally dissolves the surrounding envelopes, and on the integumental ciliary apparatus. The latter structure facilitates the passage of the animal through the "tunnel" opened by the hatching enzyme (Von Boletzky, 1979a; 1980; 1982a; *cf.* Chapters 2 to 5 of this thesis).

Although in most cephalopods the main event in hatching is the enzymatic degradation of the egg capsule, up to now the biochemical characterization of the hatching enzyme has been neglected. The present study was initiated to gather information about the European squid (*L.vulgaris*) hatching enzyme. Partial purification of the enzyme recuperated from hatching medium and some of the enzyme characteristics are described.

MATERIALS AND METHODS

Biological material

The study was carried out with embryos of *Loligo vulgaris*. Spawns were collected by fishermen from the Oosterschelde [pH 7.8 - 8 1; salinity approx. 30 ‰ (Chapters 10 and 11)] during spring and summer 1987 till 1990. At the Delta Institute for Hydrobiological Research (DIHO) in Yerseke (The Netherlands) the egg capsules were transferred within 24 h to an outdoor aquarium (capacity 1500 l) or two smaller indoor aquaria provided with running Oosterschelde water. To ensure good conditions of development the egg strings were suspended in the aquaria in a vertical position (for details see Chapter 8).

Collection of PVF

The PVF of embryos in various stages was collected from stage XII onwards. We applied the stage descriptions of Sewaga et al (1988), Arnold (1965a) and Naef (1923; 1928) to our material. The numbering of Naef was used to indicate the age of the embryos (stage XX = hatching stage)

After removal from the aquaria the eggs were placed in a dry Petri dish, cooled with melting ice. The egg capsules were broken with two watchmaker forceps. The morphology, general condition and stage of the embryos were checked From normally developed embryos the PVF was collected with a Gilson pipette and directly stored at -20°C.

Collection of crude hatching medium

As soon as the first embryos from a batch had hatched, one or more strings were gently removed from the aquarium and placed in a cuvette (10x10x6 cm) filled with 10 ml filtered (0.45 µm) Oosterschelde water. The cuvette was kept in complete darkness for at least 1 hour. During this dark period most of the stage XX embryos hatched (see Chapters 8 and 9 of this thesis). Directly after darkness the empty strings were disposed of and the crude hatching medium was separated from the hatched juveniles and egg capsule debris by filtration (Schleicher and Schuell, no. 595 $^1/_2$) The filtrate was stored at -20°C

Enzyme purification

All biochemical studies were carried out in the Department of Zoology I, Catholic University of Nijmegen, The Netherlands. The protein content of the crude hatching medium was measured by the method of Lowry et al. (1951) and by the bicinchoninic acid method of Smith et al. (1985).

Gel filtration was performed either with regular or with concentrated hatching medium. Two different methods were employed. In the first procedure in total 6 ml crude hatching medium were lyophilized and dissolved in 200 µl 0 05 M Tris-HCl buffer, pH 8 0. In the second, the hatching

medium was concentrated (30x) using Centriflo membrane cones (Type CF25, Amicon Division, W R Grace & Co). Membrane cones containing hatching medium were centrifuged for 30 min at 800 g at 4°C.

The resulting samples (200 µl regular or concentrated hatching medium) were applied to a Superose 12 HR 10/30 column connected to an FPLC system (Pharmacia LKB Biotechnology)(flow rate 0.8 ml/min; 1 ml fractions). The column was equilibrated with 0 05 M Tris-HCl buffer, pH 8 0, which was also used as elution buffer. The fractions were concentrated as described above.

Enzyme assays

The proteolytic activities of the PVF, crude hatching medium and (pooled) fractions were determined in two ways First, a fluorogenic procedure similar to that described by Barrett and Edwards (1976) was employed Equal amounts of enzyme solution and a solution of 10 mg/ml N,N-dimethylated casein in 0.1 M Tris-HCl buffer, pH 7.5, were mixed to start the reaction. Immediately after mixing a sample of 15 µl was taken and added to 500 µl 0 1 M phosphate buffer, pH 7 0 During vigorous mixing of these two solutions 500 µl of 0.3 mg/ml fluorescamine in dry acetone was added. This was also done after 5 hours incubation at 33 °C The fluorescence of the blanks (t=0), the enzyme samples (t=5) and the standards was read at lambda excitation (390 nm) and lambda emission (470 nm) on an Aminco Fluoro-Colorimeter model J4-7440. Standards of 0-100 nmol glycine in 0 1 M Tris-HCl buffer, pH 7.5, were used. One unit of enzyme activity was defined as the caseinolytic activity in 1 min resulting in the same increase in relative fluorescence as given by 1 nmol glycine.

In the second procedure Hide Powder Azure (HPA, Calbiochem) was used as chromogenic substrate. Four mg HPA were added to 1 ml sample, mixed and incubated for 10 hrs at 37°C After incubation the sample was stirred and then centrifuged for 15 min at 350 g The absorbance of the resulting supernatant was read at 595 nm. Before applying the HPA method, fractions were pooled (each pool consisted of 5 fractions), because a rather large sample volume is needed for this procedure.

Effect of pH

Fractions containing proteolytic activity were concentrated (2x) by ultrafiltration using Centriflo membrane cones To a 100 µl sample, 100 µl 0.1 M Tris-HCl buffers with various pH values were added. After mixing the pH was controlled and the proteolytic activity was determined with the fluorogenic procedure as described above

Effect of inhibitors

The effects of the following protease inhibitors (all from Merck) on *L. vulgaris* hatching enzyme were examined. phenylmethane sulfonyl fluoride (PMSF), *N*-ethylmaleimide (NEM) and ethylene diamine tetracetate (EDTA). Each inhibitor was used in two concentrations, 3.0 and 7.5 nM, in 0 05 M Tris-HCl buffer, pH 8 0. PMSF was first dissolved in methanol and this solution was diluted with buffer Final concentrations of methanol in the assay mixtures were 2 and 8%. Fifty µl of inhibitor solution were mixed with 50 µl of concentrated (2x) hatching medium, or with 50 µl fractions with proteolytic activity. The fluorogenic method was applied to measure proteolytic activity (t=0,15 and 60 min)

RESULTS

Partial purification

Figure 6 1 shows the elution profile of 30x concentrated crude hatching medium of *L vulgaris* on the Superose column and the proteolytic activity of the various fractions measured by the two different assay methods. After using the HPA method the highest proteolytic activity was found in the pool containing fractions 12 to 16 Much lower activity was found in fractions 17 to 21. These findings were in agreement with the results obtained with the fluorogenic procedure, which is more selective, *i.e* the highest proteolytic activity was found in fractions 12 and 13 while a much lower activity was detected in fractions 11, 14 and 17.

Relation to the hatching process

Crude hatching medium was obtained after embryos had hatched in 10 ml filtered Oosterschelde water. As a control this sea water was tested using both enzyme assays, but proteolytic activity was never found before the dark shock, *i.e.* before hatching took place. The protein content of crude hatching medium varied between 125 and 225 µg ml^{-1}.

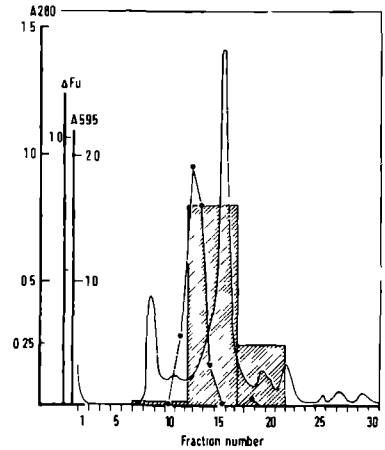

Fig. 6.1. Chromatography on Superose 12 of the water soluble proteins from the crude hatching medium of *Loligo vulgaris*. — Aborbance at 280 nm; —.— proteolytic activity determined by fluorogenic procedure (increase in relative fluorescence units). Bars = proteolytic activity measured with the chromogenic substrate Hide Powder Azure (absorbance at 595 nm).

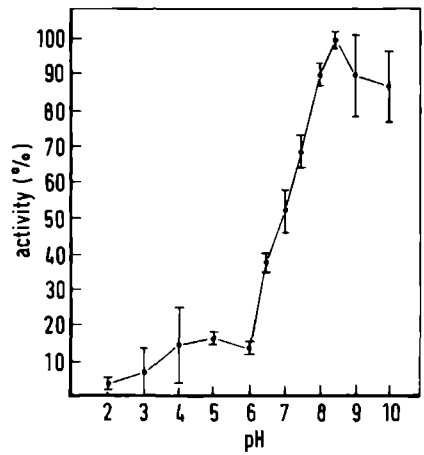

Fig. 6.2. Proteolytic activity of crude hatching medium of *Loligo vulgaris* as a function of pH. The activity was determined with the fluorogenic procedure using dimethylated casein as substrate. Mean values of relative activities from 3 experiments were plotted with standard error (bars).

Proteolytic activity was never found in PVF. The gel chromatographic pattern of PVF of stage XVIII-XX embryos was similar to that of crude hatching medium, except that fractions 12 and 13 never showed proteolytic activity.

Effect of pH and stability

The proteolytic activity of fractions 12 and 13 had a pH optimum around 8.5 (Fig. 6.2). The same results were found with crude hatching medium. The proteolytic activity of crude hatching medium was completely lost after 3 times freezing and thawing or after more than 4 months storage at -20°C. Lyophilized hatching medium kept its activity only if it was dissolved in buffer within 2-4 days. If the lyophilized medium was stored longer, proteolytic activity quickly dropped. The enzymatic activity of concentrated hatching medium was less resistant to lyophilization and storage at -20°C. Very often the activity was already lost after 24 h. In fractions 12 and 13 activity could only be found after the fractions had been concentrated on Centriflo membrane cones, and it was still detectable 1 week after storage at -20°C. Activity of this material was completely abolished by lyophilization.

Effect of inhibitors

Protease activity of crude hatching medium and of fractions 12 and 13 was almost entirely lost after treatment with the metal chelator EDTA (for percentages see Table 6.I). Less inhibition was found after exposure of the enzyme to PMSF or to NEM (see Table 6 I).

DISCUSSION

The pH optimum of the *L.vulgaris* protease in hatching fluid, *viz.* 8 5, is proximate to the pH of natural sea water (*e.g.* pH 7.8 - 8.1 for the Oosterschelde). Investigations of D'Aniello et al (1989) have shown that *L vulgaris* embryos develop and hatch normally at pH values between 7.8 and 8.4. Beyond this range disturbances occurred. During embryonic development the ionic concentration in the PVF remains in equilibrium with the surrounding sea water (D'Aniello et al., 1986, De Leersnyder and Lemaire, 1972).

Studies on hatching enzymes from various teleosts living in fresh water, brackish water and sea water show that the pH optima of these enzymes are also relatively high (pH 7-9) (Denucé, 1976; Denucé and Thijssen, 1975; DiMichele et al., 1981; Hagenmaier, 1974; Oppen-Berntsen, 1990, Schoots and Denucé, 1981, Yamagami, 1970; 1973). Similar values were obtained for the hatching enzyme of the amphibians *Rana chensinensis* and *Xenopus laevis* (pH optima around 7.7)(Katagiri, 1975; Urch and Hedrick, 1981), the hatching enzymes of various echinoderms (pH 7.5 - 9.5) (review in: Denucé, 1984; 1991) and tunicates (pH 7-10) (review in. Denucé, 1984; 1991). Only the ovisacase of the barnacle *Balanus balanoides* stands in marked contrast to most other hatching proteases investigated so far, in having optimal activity in the acidic range (pH 3.0)

Table 6.I. Effect of inhibitors on the proteolytic activity of crude hatching medium of *Loligo vulgaris.*

inhibiting reagent	concentration (nM)	reduction of activity (%)
EDTA	3.0	91 2
	7.5	84.3
PMSF	3.0	41.7
	7.5	45.4
NEM	3 0	58.8
	7.5	43.3

(Barnes and Blackstock, 1977)

The present inhibitor studies suggest that the hatching fluid of *L.vulgaris* contains a metalloprotease. Metalloproteases functioning as hatching enzymes are found in many vertebrate and invertebrate species (Denucé, 1984; 1991). For example, metal analysis on isolated pike hatching enzyme (*Esox lucius*) demonstrated that the enzyme is a zinc-metalloprotease (Schoots and Denucé, 1981). On the other hand, the hatching enzymes of the teleosts *Fundulus heteroclitus* and *Brachydanio rerio* are probably serine proteases (Denucé and Thijssen, 1975; DiMichele et al., 1981).

In the course of the present study proteolytic activity was never encountered in the PVF. These results support the assumption that the hatching enzyme of *L.vulgaris* is released just before and during hatching (Denucé and Formisano, 1982;see also Chapters 3 to 5 of this thesis). These findings are also in agreement with ultrastructural investigations of the chorion of *L.vulgaris* before and after hatching. Scanning electron microscopy clearly indicated that the chorion was only digested in a restricted area during hatching, while the remnants were not affected by proteolytic action (M.E.F. Roozen, unpublished results). It seems that in *L vulgaris* the hatching enzyme is used in a highly efficient manner. Chances that the embryo itself is damaged by the hatching protease are limited. This in contrast to various teleost embryos in which hatching is preceded by a first proteolytic attack on the chorion which causes swelling of the egg shell (Ogawa and Ohi, 1968; Ohi and Ogawa, 1970; Schoots et al., 1983c, Yasumasu, et al , 1989a; 1989b)

During this study it was found that purification of *L.vulgaris* hatching enzyme is hampered by the presence of a haemagglutinin in the PVF. The latter has been described by Marthy (1974). Other circumstances which impaired further purification were the scarcity of the material, the instability of the enzyme which necessitates the handling of the enzyme in a rather brief period of time, and the low quantity of enzyme contained in the hatching fluid, because in *L.vulgaris* usually not all the enzyme is being released from the gland during hatching (Chapter 3 of this thesis).

Because high amounts of hatching enzyme reside in Hoyle's organ, it would be obvious to use this gland as starting material for the purification, provided the enzyme is not stored in an inactive form. Unless Hoyle's organ is excised neatly and free from adjacent tissues, one must take into account that extracts can contain other proteases, such as the proteases of the yolk syncytium, the digestive organs and the mantle muscle, which will probably show proteolytic activity during late embryonic development and are localized in the distal end of the embryo (Arnold, 1968; 1971; Fioroni, 1978; Meister, 1972; Sakai and Matsumoto, 1981; Sakai-Suzuki, et al., 1983; 1986). It is also important to avoid the interference of protease-inhibitors which are present in several organs of squids (Tschesche and Rucker, 1973a; 1973b; 1974).

To further purification purposes, it seems mandatory that the hatching enzyme can be easily distinguished from other proteases. Pauli et al. (see Chapter 5 of this thesis) have succeeded in "staining" the granules in the cells of Hoyle's organ The specific carbohydrate nature of these granules could be visualized by using labeled lectins. Lectins are not only suitable as histochemical markers but can also be used in biochemical investigations (Goldstein and Hayes, 1978). In the case of the squid hatching enzyme the use of lectins which attach specifically to the carbohydrate moiety of the enzyme deserves particular attention in further purification procedures.

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The authors are grateful to Mr P.L. Pascoe (Plymouth Marine Laboratory, U K) and Mr. R.H. Bogaards (Delta Institute for Hydrobiological Research in Yerseke, The Netherlands) for collecting biological material. We also want to thank Mrs. M.E F. Roozen and Mrs. J.A.J.M. van den Hurk for practical help, Mr. C.M. de Rooy for taking care of the animals and Mrs. E.A.J. Derksen for secretarial assistance.

PART II: Environmental aspects

THE EFFECT OF COPPER ON THE EMBRYONIC DEVELOPMENT AND HATCHING OF *SEPIA OFFICINALIS*

ABSTRACT. The influence of copper on embryonic development and hatching of *Sepia officinalis* was investigated. Copper exerts a profound effect on both hatching stage and time-to-hatching At high copper concentrations (50-200 ppb), the embryos hatch at an earlier stage than the controls, but they have a lower survival potential No external morphological malformations were found. Whereas copper does not accumulate in the embryo or in the vitellus, it is absorbed by the envelope and/or chorion.

INTRODUCTION

Copper is an essential trace element for many biological processes. The need for copper in cephalopod embryos and its potential toxic effects on development and hatching have, thus far, received scant attention. *In vivo* studies on molluscan haemocyanins demonstrated that copper is essentially involved in the biosynthesis of this hemolymph pigment, which is present in cephalopods, amphineurans and many gastropods, but not in bivalves (Ghiretti et al., 1959, Manwell, 1960; Schmidt-Nielsen, 1975) Distribution studies have indicated that in cephalopods, besides the hemolymph, the hepatopancreas is the richest in copper (Ghiretti and Violante, 1964; Schipp and Hevert, 1978). The alimentary copper is stored in the hepatopancreas and utilized for biosynthesis of haemocyanin throughout life (Ghiretti and Violante, 1964).

Eggs of the cuttlefish *Sepia officinalis* develop in delicate follicles which are traversed by blood vessels (Tompsett, 1939). Transfer of haemocyanin and other proteins takes place from the maternal hemolymph to the developing oocytes by cells of the follicular epithelium (Konopacki, 1933) As a first approach to the study of copper metabolism in *S.officinalis*, Decleir et al. (1970) measured the total copper content in embryos and newly hatched individuals. They concluded that the total amount of copper does not change during embryonic life. Copper is found in the yolk sac of very young embryos and is subsequently transferred to the embryo proper. The embryonic development of the cuttlefish was first described in detail by Koelliker (1844). The description of the various stages has been complemented by Naef (1928) and Lemaire (1971). Lemaire distinguishes 30 stages in the normal embryonic development of *S.officinalis*. The stages following organogenesis take more than half of the total length of embryonic development (Von Boletzky, 1983).

Freshly laid eggs of *S.officinalis* consist of a black coloured envelope, a chorion, a thin layer of perivitelline fluid and yolk covered by a thin layer of ooplasm, which, after fertilization, undergoes discoidal cleavage, leading to the formation of a blastodisc. With proceeding embryonic development, the still elastic envelope is dilated by the chorion which expands because of the osmotic pressure of the perivitelline fluid (Lemaire, 1971) Finally, the embryo hatches by locally dissolving the chorion and squeezing the body through the hole thus produced in the envelope (Von Orelli, 1959). Under stress conditions, embryos may hatch prematurely and show a still sizable outer yolk sac (Fioroni, 1978).

Chapter 7 is the modified version of Paulij, W P , Zurburg, W , Denucé, J M , Van Hannen, E J (1990) The effect of copper on the embryonic development and hatching of *Sepia officinalis* L Arch Environ Contam Toxicol 19 797-801 Contribution no 471 of the Delta Institute for Hydrobiological Research

To measure the effects of copper on embryonic development and hatching of the cuttlefish, embryos were exposed to a range of copper concentrations

MATERIALS AND METHODS

The study was carried out at the Delta Institute for Hydrobiological Research with eggs of *S.officinalis*, which were collected from the Oosterschelde (South Western part of The Netherlands) or from sexually mature specimens kept in sea water tanks. Two spawns were used for the various experiments. The first spawn was laid on April 30, 1988 in an outside aquarium (1500 l). The aquarium was provided with running sea water coming directly from the Oosterschelde (salinity see Chapters 10 and 11). On June 2, 1988, when the embryos had reached developmental stage 8 to 10 according to Lemaire (1971), the eggs were removed from the aquarium to be used in Groups 1A to 1D (experiment 1). The material used in experiment 2 was collected by fishermen on July 2, 1988. The eggs were kept in the outside sea water aquarium until July 6, 1988. At the time they were used in experiment 2, the embryos had reached developmental stage 23 to 25 (Lemaire)

Both experiments were performed in four equally sized perspex aquaria (capacity 20 l), containing 12 l of filtered Oosterschelde sea water (Millipore; filter pore size 0.45 µm) and placed in an automatic controlled environmental chamber at 15°C. To facilitate the study of development and hatching, a floating system was applied in each aquarium consisting of 12 perforated plastic tubes (diameter 4.5 cm)(cf. Chapter 10). Four embryos were placed in each tube: the experiments always started with 4 x 48 embryos. Each aquarium was connected to an individual pumping device which enabled a closed circulation system without the need for extra aeration.

The first aquarium (Group A) containing 12 l of filtered sea water served as control, while in the other aquaria the copper concentration was increased by adding cupric chloride ($CuCl_2$) to about 50, 100 and 200 ppb Cu^{2+}, in aquarium B, C and D, respectively. At intervals of 7 days, the sea water was removed and replaced by water containing the appropriate amount of copper. The actual amount of free copper in the sea water was measured on the first and the seventh day with a Chemotronics portable digital voltmeter (PDV 2000) without any additional treatment.

Tissue samples were taken on the first and subsequently every seventh day. From each aquarium, four eggs from four different tubes were taken. In experiment 1 the eggs were dissected to produce envelope with chorion, vitellus and embryo. As in stage 1 to 20 embryos it is almost impossible to separate the vitellus from the embryo, the total copper content of vitellus plus embryo was measured. In experiment 2, four whole eggs were sampled. The tissue samples were freeze-dried followed by destruction in a mixture of HNO_3, HCl and H_2O (8:2:2). The total amount of copper was measured by atomic absorption spectroscopy (Perkin-Elmer Zeeman 3030 instrument).

The hatching percentage as well as the hatching date were recorded. All hatched juveniles were fixed in Hollande's fixative (Gurr, 1962) for the microscopic study of possible external morphological malformations as a result of the exposure to extra copper. The results were analyzed by SYSTAT (Wilkinson, 1988). The following tests were applied: linear analysis of covariance, likelihood ratio test for RxC tables and the ANOVA and Tukey HSD multiple comparison *a posteriori* test.

RESULTS

The free copper concentrations in the aquaria during the experiment are given in Fig. 7 1. The normal copper value for Oosterschelde water (which was used as a reference) is approximately 4 ppb (Valenta et al., 1984, Revis et al., 1989) The percentages of hatched embryos are given in Figs. 7 2a and b. On the average 55% of the embryos hatched. In experiment 1, there was no significant difference between the hatching percentages of the experimental groups (likelihood ratio test for RxC tables, $p=0.808$), in contrast to experiment 2 ($p<0.001$). This low probability is mainly caused by the high hatching percentage in Group 2C The results of the Groups 2A, 2B and 2D are in agreement with the results of experiment 1 and do not confirm the percentages of Group 2C.

Copper strongly influenced the timing of hatching of *S.officinalis* embryos (Figs. 7.3a and b). In experiment 1, the length of time necessary to accomplish development was significantly different in different copper concentrations (ANOVA test on development times, p<0 001). The Tukey multiple comparison *a posteriori* test shows significant differences between all pairs of groups (p<0.001). In experiment 2, only Groups 2A and 2B were not significantly different (p=0.074), all other groups showed, similar to groups in experiment 1, significant differences in the duration of development (p<0.001).

Not only the timing of hatching, but also the stage in which the embryos hatch was affected by copper (Figs. 7.4a and b). Normally, embryos hatch in developmental stage 30 (Lemaire, 1971). Occasionally, less than 5% of the embryos show premature hatching prior to stage 30 (Fioroni, 1978). With increasing free copper concentrations of the sea water, more embryos hatched prematurely (Figs. 7.4a and b). No external morphological malformations were observed. Both

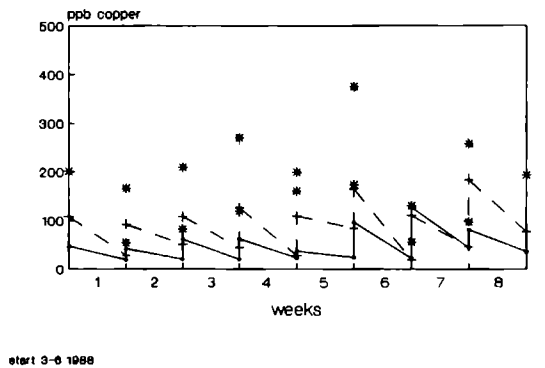

Fig. 7.1. Free copper concentrations in the various aquaria to which *Sepia officinalis* eggs were exposed. —— =B, —+— =C, ...*... =D.

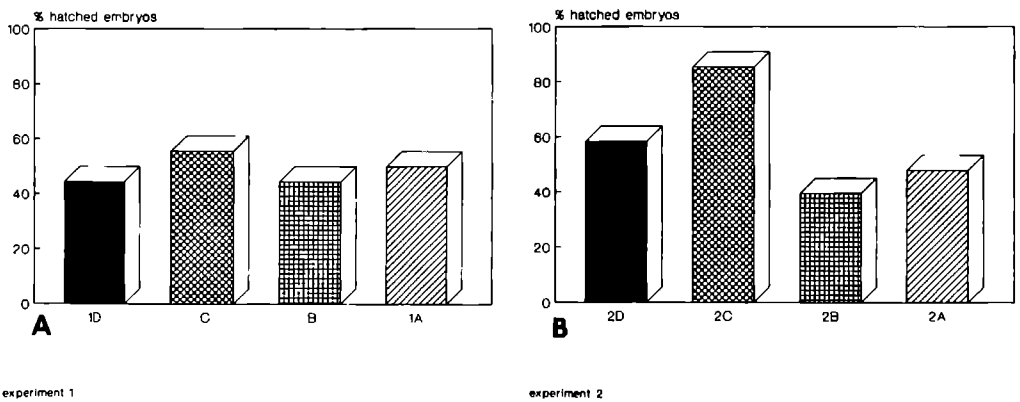

Fig. 7.2. Percentage hatching of *Sepia officinalis* exposed to different copper concentrations. Group 1A and 2A are controls For copper concentrations of Group 1B to 1D and 2B to 2D see Fig. 7·1. For easier comparison of this and the following figures the groups were ranged from left to right in order with the events occurring in the experimental period, as shown in Fig. 7.3.

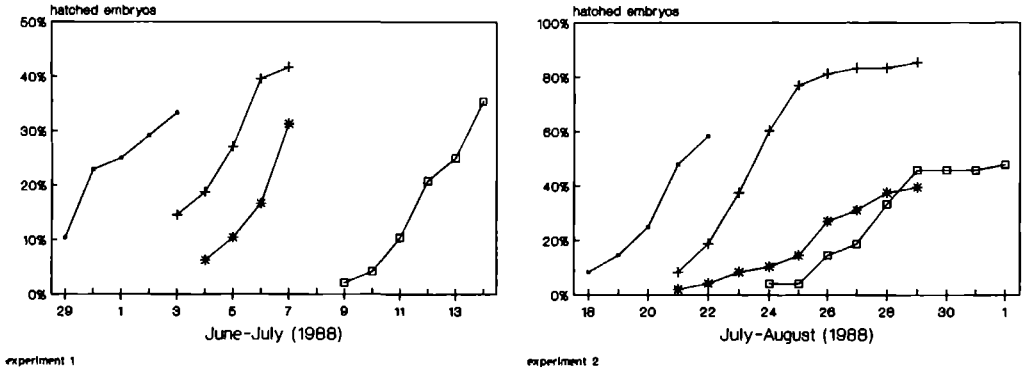

Fig. 7.3. Influence of copper on the hatching time of *Sepia officinalis* embryos. ■ =D, + =C, * =B and [] =A.

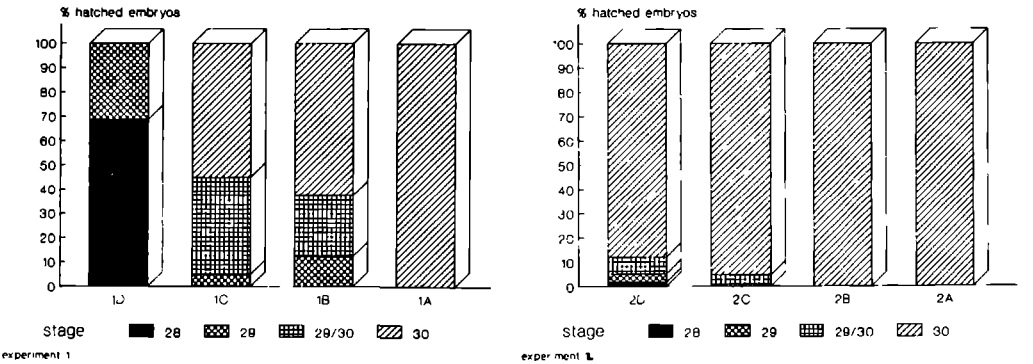

Fig. 7.4. Influence of copper on the hatching stage of *Sepia officinalis* (staging system of Lemaire, 1971).

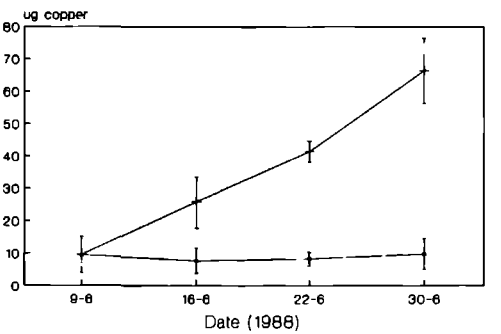

Fig. 7.5. Copper accumulation (in µg copper per envelope/chorion) in the envelopes and chorions of *Sepia officinalis* eggs. ◆ =Group 1A, + = Group 1B. For explanation of 1A and 1B see Figs. 7.1 and 2.

Table 7.I. The total copper amount (µg) in embryos plus vitellus of *Sepia officinalis* in experiment 1 and the amount in early juveniles of groups 2A to 2C.

Group	Embryo + vitellus	Group	Juveniles
1A	2.8 ± 0.2	2A	5.2 ± 2.4
1B	2.7 ± 1.0	2B	5.4 ± 2.8
1C	3.0 ± 0.6	2C	4.7 ± 3.0
1D	4.9 ± 4.0		

experiments showed differences in distribution of the embryonic stages in which the embryos hatched (likelihood ratio test for RxC tables, $p<0.001$).

At the highest copper concentrations of experiment 1 (Group 1D), all embryos hatched before reaching stage 30. The embryos which succeeded to stage 30 were small and still provided with big yolk sacs, but the mantles had a normal red-brownish colour. In the control Group 1A, all embryos reached stage 30 and hatched with small external yolk sacs. Juveniles which hatched in Group 1D and were left in the aquarium never lived longer than 12 hrs. Even directly after hatching the juveniles were not able to swim horizontally; instead, they hung vertically in the water. In the controls, newly hatched juveniles immediately hid themselves on the bottom of the tubes and attacked small objects in the water. These juveniles invariably lived longer than 36 hrs. Whereas the fry in Group 1D looked pale and whitish, early juveniles in the controls displayed a normal red-brownish colour and chromatophore expansions. Also, in experiment 2, most premature hatchlings were found in aquarium D, but the vast majority of the embryos reached stage 30. As noted for experiment 1, none of the juveniles in Group D lived longer than 12 hrs while in Group 2C, 2B and 2A all fry stayed alive for more than 36 hrs.

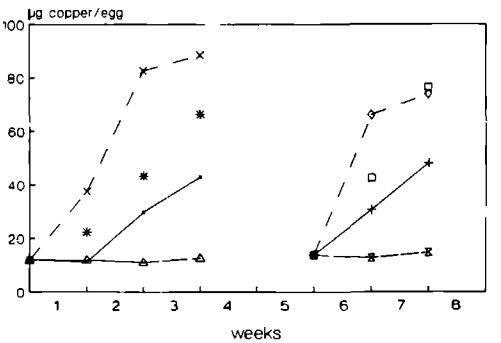

Fig. 7.6. Copper accumulation (in µg) in eggs of *Sepia officinalis*. Left part: experiment 1 (Δ =1A, ■ =1B, ...*... =1C and —x—= 1D. Right part: experiment 2 (_ $\frac{7}{0}$ _ =2A, _+_ 2B, ...[]... 2C and — ◊ — =2D).

In experiment 1, the total amount of copper in embryo plus vitellus showed no difference between the groups (see Table 7 I) Also in experiment 2 there was almost no difference in early juveniles. In contrast, the amount of copper in the chorion and/or the envelope of developing embryos increased in the copper exposed Group 1B, compared to the controls (linear analysis of covariance, $p<0.001$)(Fig. 7.5).

In the control group, the copper content of whole eggs remained constant during the entire experiment (Fig. 7 6). In all other experimental groups, the content increased as copper concentration in the water increased (linear analysis of covariance, $p<0.001$).

DISCUSSION

At present, our knowledge of the effect of micropollutants on the embryonic development in cephalopods is very scarce. The effects of pollutants on hatching and development of fish have been investigated more extensively Rosenthal and Sperling (1974) found that exposure of eggs of Atlantic herring to cadmium shortened the incubation period by 1 (0.1 ppm Cd) to 5 (10 ppm Cd) days Similar findings were obtained for gar pike eggs (Westernhagen et al., 1975).

Our results indicate that copper exerts a profound effect on timing of hatching in the cuttlefish. At higher copper concentrations, the embryos hatch earlier but have a lower survival potential probably because of premature hatching. It is also possible that the copper in the sea water affects the fry since they are no longer protected by the envelope. The effect of copper on the timing of hatching is most pronounced when copper is added from early embryonic development on. The hatching percentage of *S.officinalis* is probably not affected. It is not likely that the high percentage in Group 2C is caused by copper and the rest of the groups do not show significant differences.

It was observed that during embryonic development the total amount of copper in the embryo plus vitellus did not change (Table 7.I). A similar result was obtained by Decleir et al. (1970). These authors reported a level close to 3.8 µg per embryo plus vitellus, whereas we found a level of approximately 3 0 µg. The same copper contents were found for embryos from the different experimental treatments. From these findings, it is concluded that there was no detectable uptake of copper from the sea water by the embryo during embryonic development. These results disagree with Ranzi (1938), who states that copper in embryos of *S.officinalis* has its orgin in the surrounding sea water and accumulates as development proceeds.

Early animal development takes place inside a fluid-filled chamber, enclosed by a protective acellular structure or egg envelope. Most cadmium contamination was found on the outer surface of the capsule of Atlantic herring eggs (Rosenthal and Sperling, 1974). It was suggested that short-term exposure of herring eggs to heavy metals can change the properties of the egg surface. These changes were considered as sublethal responses; they can alter the organization within the egg in such a way that a significant reduction in survival potential of the embryo may occur in later stages of embryonic development. In fathead minnow embryos, zinc has an effect on the strength of the chorion. The force required to rupture the chorion seems to be inversely related to the toxicant exposure concentration (Benoit and Holcombe, 1978; Holcombe et al., 1979). Rosenthal and Alderdice (1976) concluded that fertilized marine fish eggs are well protected against heavy metal pollutants for which the envelope and jelly coat act as a barrier to prevent penetration.

In cephalopod eggs, the chorion and egg envelope form the chamber in which embryonic development takes place. During its development the embryo is in direct contact with the perivitelline fluid (PVF) which is continuously hypertonic to sea water (Lemaire, 1971). In our experiments, the chorion and the envelope were formed before the egg came into contact with the pollutant. Because we found that the total copper amount in developing embryos of *S.officinalis* remained constant, we conclude that the envelope and/or chorion (as in marine fish eggs) protects the developing embryo against high external copper concentrations. From observations of the controls and Group 1B, it can be concluded that copper accumulates in the envelope and/or the chorion.

The envelope primarily consists of several jelly layers. At the moment of hatching, due to the expanding PVF, the envelope and the chorion are stretched and become rather thin (Lemaire, 1971) It is possible that copper changes the physico-chemical properties of the envelope and/

or the chorion Moreover, in late embryonic stages the protection by either one could decline. The concentration of copper in the PVF increases to a point that copper affects the embryo causing premature hatching. This explains copper influence on hatching time

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THE IMPACT OF PHOTOPERIODICITY ON HATCHING OF *LOLIGO VULGARIS* AND *LOLIGO FORBESI*[*]

ABSTRACT. The influence of photoperiodicity on hatching of *Loligo forbesi* and *Loligo vulgaris* embryos was investigated under different experimental light-dark (LD) conditions. The transition from light to dark stimulated hatching and functions as a "Zeitgeber" or synchronizer. Independent of the timing and duration of the dark period most embryos hatched soon after termination of the light period. Embryos which had developed in continuous light, showed no hatching rhythm at all. When exposed to a dark shock most embryos hatched soon after the onset of darkness. A twilight shock, in which the light was reduced by 50% (*i.e.* 50 $\mu E\ s^{-1}\ m^{-2}$), failed to stimulate hatching. Embryos which were kept from stage X on in an artificially controlled LD cycle, preferentially hatch in a period which coincides with the period at which darkness usually occurred when placed in continuous illumination from stage XX onwards.

INTRODUCTION

The influence of extrinsic factors, such as light, on hatching of cephalopod embryos has so far only been dealt with sporadically, in contrast to other zoological groups, *e.g.* fishes and insects. This holds in particular for the species *Loligo forbesi* Steenstrup, 1856 (veined squid) and *Loligo vulgaris* Lamarck, 1798 (European squid). *L.vulgaris* and *L.forbesi* eggs have quite translucent egg-capsules and are deposited generally on a solid substrate in relatively shallow water (Fioroni, 1978).

In the summer *L.forbesi* spreads throughout the English Channel and Southern North Sea, particularly in coastal waters. In October there is a distinct off-shore movement and the animals tend to occupy the Western Channel. The main spawning period for *L.forbesi* at Plymouth occurs in winter when the temperature of the sea water is about 9-11°C (Holme, 1974; Southward, 1960). *L.vulgaris* is one of the most common squids along the north-eastern Atlantic and Mediterranean coasts (Worms, 1983). The main spawning period for *L.vulgaris* occurs in spring. Temperature strongly influences the embryonic developmental rate (Roper et al., 1984).

The egg strands of *L.forbesi* are very similar to those of *L.vulgaris* (Von Boletzky, 1987b). In eggs obtained by natural spawning the chorions start to expand halfway through the embryonic development due to the increasing amount of perivitelline fluid (PVF). Therefore, in advanced stages the embryo is able to move freely within the greater volume of PVF. Our own observations indicate that the hatching behaviour of *L.forbesi* and *L.vulgaris* starts with strong contractions of the mantle and swimming around of the embryo while the epidermal hatching gland, the so-called organ of Hoyle (Hoyle, 1889), rubs against the chorion. Consecutively, the animal starts bumping against the chorion while showing chromatophore expansions. Finally, the embryo hatches by locally dissolving the chorion and squeezing the body through the hole thus produced in the envelope (Von Orelli, 1959). There is circumstantial evidence that a protease originating in the

[*] Chapter 8 is the modified version of: Paulij, W.P., Herman, P.M.J., Van Hannen, E.J., Denucé, J.M. (1990). The impact of photoperiodicity on hatching of *Loligo vulgaris* and *Loligo forbesi*. J. mar. biol. Ass. U.K. 70: 597-610. Contribution no. 487 of the Delta Institute for Hydrobiological Research.

organ of Hoyle is acting as a hatching enzyme (Denucé and Formisano, 1982).

The objective of the present study was to collect data on how the hatching of *L.forbesi* and *L.vulgaris* is affected by various light conditions.

MATERIAL AND METHODS

The study was carried out at the Plymouth Laboratory of the Marine Biological Association (MBA), United Kingdom, in 1987 and at the Delta Institute for Hydrobiological Research (DIHO) in Yerseke, The Netherlands, in 1988 and 1989, with embryos of *Loligo vulgaris* and *Loligo forbesi*. Eggs of *L forbesi* were collected from the English Channel. At the MBA, the spawns were moved within

Table 8.I. Schedule of experiment 1

Group	Species	N	LD Cycle
1:1	*L.forbesi*	126	LD 11:13 (D 19.00-08.00 hr)
1.2	*L.forbesi*	503	LD 13 11 (D 20.00-06.00 hr)
1:3	*L.vulgaris*	677	LD 13:11 (D 20.00-06.00 hr)
1:4	*L.forbesi*	348	LD 22:2 (D 12.00-14.00 hr)
1:5	*L.forbesi*	366	LL (constant light)
1:6	*L.vulgaris*	498	LL (constant light)

24 hrs of collection into an indoor aquarium with running sea water. In 1988 and 1989 *L.forbesi* egg masses in very early developmental stages were sent to the DIHO.

L.vulgaris eggs were collected by fishermen from the Oosterschelde during spring and summer of 1988 and 1989. At the DIHO the egg capsules were moved within 24 hrs into an outdoor aquarium (capacity 1500 l) provided with running sea water. To ensure good conditions of development the egg strings were suspended vertically in the aquaria. When the strings were deposited on the bottom of the aquarium almost half of the total number of embryos died, especially the ones in direct contact with the bottom, probably due to lack of oxygen.

Experiments started when more than 80% of the embryos in one string had reached stage X. We applied the stage descriptions of Segawa et al. (1988), Arnold (1965a) and Naef (1923; 1928) to our material. The numbering of Naef was used to indicate the age of the embryos.

Experiment 1 is summarized in Table 8.I. Switchings between light and darkness were abrupt, without gradual transition. For Group 1:1 two *L.forbesi* strings from two different spawns were used. These spawns were laid on 10 and 15 November 1987. When the embryos had reached stage X the strings were moved into a smaller aquarium (15 l) with circulating sea water. An artificially controlled LD cycle was installed. The light intensity, measured with a Metrawatt meter (model no. Tavolux 2 PL X) with a platinum opal filter, was 130 $\mu E\ s^{-1}\ m^{-2}$ during light periods and 0 $\mu E\ s^{-1} m^{-2}$ during darkness. In Group 1:1 the water temperature fluctuated between 12.0 and 17.0 °C (mean=15.2 ± 1.3 °C). There was also a temperature variation between day and night of 0.6 - 3.1 °C.

Table 8.II. Schedule of experiment 2.

Group	N	LD cycle from stage X to XX	LD cycle from stage XX on
2:1	84	LD 22:2*	LD 22:2*
2:2	63	LD 22:2*	LL (constant light)
2:3	95	LD 22:2*	2 dark periods: 08.00-10.00 hr, 12.00-14.00 hr
2:4	64	LD 22:2*	3 dark periods: 08.00-10.00 hr, 12.00-14.00, 16.00-18.00 hr

* dark 12.00-14.00 hr

The remaining part of experiment 1 (and experiments 2 to 4) were carried out at the DIHO in automatically controlled climate chambers kept constantly at a temperature of 15 °C. The temperature of the water and the air was continuously monitored to ensure that the light did not heat up the water. The light intensity measured as mentioned above was 100 $\mu E\ s^{-1}\ m^{-2}$ in light and 0 $\mu E\ s^{-1}\ m^{-2}$ in dark periods. In Groups 1:2 to 1:6, *L.vulgaris* and *L.forbesi* embryos were exposed to several artificial LD cycles from stage X on. As soon as the embryos had reached stage XX (*i.e.* hatching stage), the egg-strings were placed in cuvettes (10x10x20 cm). In this embryonic stage the external yolk is almost absorbed (approximately equal to or less than the length of arms II) and only the yolk sac envelope remains (Segawa et al., 1988). Juveniles were collected directly before and after the onset of darkness. In Groups 1:5 and 1:6, kept in constant light, juveniles were collected at 06.00 and 20.00 hr.

For experiment 2 only *L.vulgaris* embryos were used. All embryos developed from stage X on in a LD cycle of 22:2 hr, being dark between 12.00 and 14.00 hr (comparable with *L. forbesi* Group 1:4). In Group 2:1 (control) the embryos remained under the aforementioned conditions, while in all other groups the LD cycle was changed after 80% of the embryos had reached stage XX. The treatments are summarized in Table 8.II. Juveniles were counted every two hours.

In experiment 3 *L.vulgaris* embryos developed in constant light until the majority had reached stage XX. Group 3:1 was the control. The stage XX embryos in all other groups were exposed to various twilight and/or dark periods (Table 8.III). In Group 3:6 and 3:7 embryos were exposed to a twilight period. During this period the light was reduced by 50% (50 $\mu E\ s^{-1}\ m^{-2}$). In experiment 3 juveniles were counted every hour or every two hours but never during dark or twilight periods.

In experiment 4 *L.vulgaris* and *L.forbesi* embryos developed in a LD cycle of 12:12 hr (light started at 08.00 hr) or in constant light and remained under these conditions during the whole experiment. Juveniles were counted every two hours. In order to count the juveniles during darkness, the egg strings were placed in cuvettes after the majority in a string had reached stage XIX (the external yolk sac approximately equal in length to tentacle length). Every two hours and during light and dark periods, the egg strings were placed gently in a similar cuvette with sea water of exactly the same temperature. During dark periods this was carried out in complete darkness. The cuvettes containing the juveniles were taken out of the climate chamber and were counted (N=89). To ensure that this treatment had no effect on embryonic development and hatching rhythm, another experiment was conducted were 90 embryos were treated as above but remained in constant light.

To visualize the effect of light on the hatching rhythm of *L.vulgaris* and *L.forbesi*, specially devised time-lapse photographic equipment was placed in a separate climate chamber. Embryos which developed in the same 12:12 LD cycle as in experiment 4 were used. When 80% of the embryos

Tabel 8.III. Schedule of experiment 3.

Group	N	Stage X-XX	LD conditions from stage XX on
3:1	190	constant light	LL (constant light)
3:2	153	constant light	one dark period 08 00-12.00 hr, followed by constant light
3:3	87	constant light	one dark period 10 00-11.00 hr, followed by constant light
3:4	147	constant light	one dark period 08.00-08.10 hr, followed by constant light
3:5	167	constant light	from 08.00 hr on, repeated one hour dark and one hour light periods
3:6	77	constant light	50% light reduction ("twilight period")08.00-09.00 hr,darkness 10.00-11.00 hr, followed by constant light
3:7	109	constant light	50% light reduction ("twilight period")08.00-10.00 hr,darkness 12 00-14.00 hr, followed by constant light

had reached stage XVII, the egg strings were divided into small pieces and the outer jelly layer was removed. The egg fragments were placed in a cuvette with four compartments for further development. The LD conditions were not changed. The water was renewed twice a day. As soon as the first embryos reached stage XX the cuvette was placed under the camera, a few hours before darkness. A picture was made one minute before and 15 minutes after the dark period started, using a far-red flash light during darkness.

A G/q single classification goodness-of-fit test (Sokal and Rohlf, 1981, p.705) was used to test if hatching was distributed over time periods according to intrinsic null hypothesis. An RxC test of independence using G/q (Sokal and Rohlf, 1981, p.745) was used to test homogeneity of different data sets.

RESULTS

The results of experiment 1 are given in Fig. 8.1. In Group 1:1 none of the 110 juveniles counted hatched during light periods (Fig. 8.1A). This is highly significant (exact binominal test, $p<0.001$). Hatching was spread over eight 24 hrs cycles. The highest hatching percentage was found during the third dark period. The spreading of hatching over several dark periods can be explained by the fact that the distal eggs in a strand developed faster (2 to 4 days) than the proximal ones. Similar observations were made with spawns of *L.vulgaris*.

In Groups 1.2 to 1.6 (Figs 8.1B to F) the temperature of the water was kept constantly at 15 °C, in contrast to Group 1:1 where the temperature fluctuated during development and hatching. In Groups 1:2 to 1:4 no temperature fluctuations were measured during and between light and dark periods (15°C). In Groups 1:2 and 1·3 (Figs 8.1B and C) the light period started and

ended at 06.00 and 20.00 hr, respectively, and hatching during light periods differed significantly from hatching during darkness (G/q test H_o:L=D in each 24 hr cycle, $p<0.0005$). The highest hatching percentage in both groups was observed during darkness, as in Group 1:1, although some hatching also occurred during light.

In Group 1:4 (Fig. 8.1D) *L.forbesi* embryos were exposed to an LD cycle with a very short dark period from 12.00 till 14.00 hr. In Group 1:4 the highest hatching percentage was also found during darkness. The total amount of hatching during the five dark periods differed significantly from the amount of hatching during all light periods (G/q test H_o: hatching per hour is equal in L and D, $p<0.0005$).

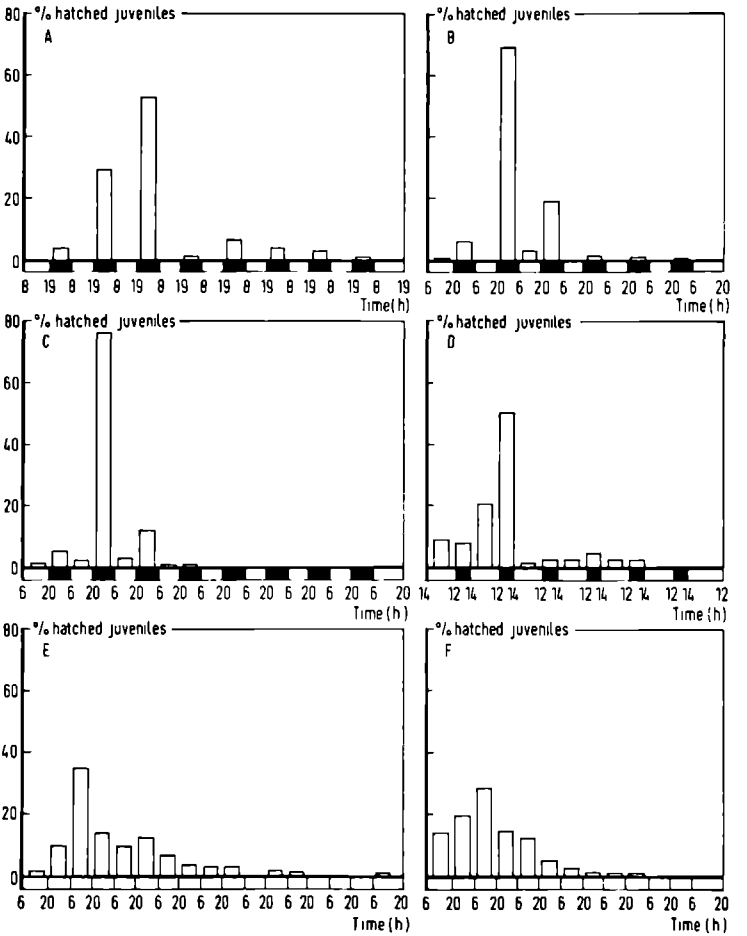

Fig. 8.1. The effect of LD periods and constant light on the hatching rhythm of *Loligo forbesi* and *Loligo vulgaris* embryos. Horizontal bar: white=light period, black=dark period. A = *Loligo forbesi* Group 1:1. Temperature fluctuations between day and night were noticeable. B = *Loligo forbesi* Group 1:2. C = *Loligo vulgaris* Group 1:3. D = *Loligo forbesi* Group 1:4. E = *Loligo forbesi* Group 1:5 and F = *Loligo vulgaris* Group 1:6. Embryos of both last mentioned groups developed and hatched in constant light. Embryos were counted at 06.00 and 20.00 hr. In Group 1:2 to 1:6 the temperature was kept constant at 15°C. The light intensity during illumination was 100 $\mu E\ s^{-1}\ m^{-2}$ and during darkness 0 $\mu E\ s^{-1}\ m^{-2}$.

In Groups 1:5 and 1:6 (Figs. 8.1E and F) embryos of both *L.forbesi* and *L.vulgaris* developed in continuous illumination. Juveniles were counted at 06.00 and 20.00 hr so that the hatching rhythm of the Groups 1:2, 1:3, 1:5 and 1:6 could be compared. In Groups 1:2 and 1:3 hatching almost always occurred between 20.00 and 06.00 hr, *i.e.* the dark period. In Groups 1:5 and 1:6 in some cases hatching was significantly higher between 06.00 and 20.00 hr and sometimes between 20.00 and 06.00 hr. Apparently, embryos in these two last-mentioned groups hatched as soon as their development was accomplished, completely independent of any period within the 24 hrs cycle.

For experiment 2 only *L.vulgaris* embryos were used and the results of this experiment are given in Fig. 8.2. In this figure all juveniles counted are represented by compiling hatched fry within 2 hr periods. In Group 2:1 (Fig. 8.2A) hatching was spread over five 24 hrs cycles. These embryos developed from stage X on in a LD cycle of 22:2 hrs, as did the *L.forbesi* embryos of Group 1:4. The amount of hatching during the dark periods differed significantly from hatching during light (G/q test H_o: equal probability of hatching in all 2 hr periods, $p<0.0005$). Most embryos in Groups 1:4 and 2:1 hatched during the short dark period.

In Group 2:2 the dark period was eliminated as soon as the embryos reached stage XX (Fig. 8.2B). The hatching rhythm in Group 2:2 differed significantly from Group 2:1 (*i.e.* control) ($p<0.0005$).

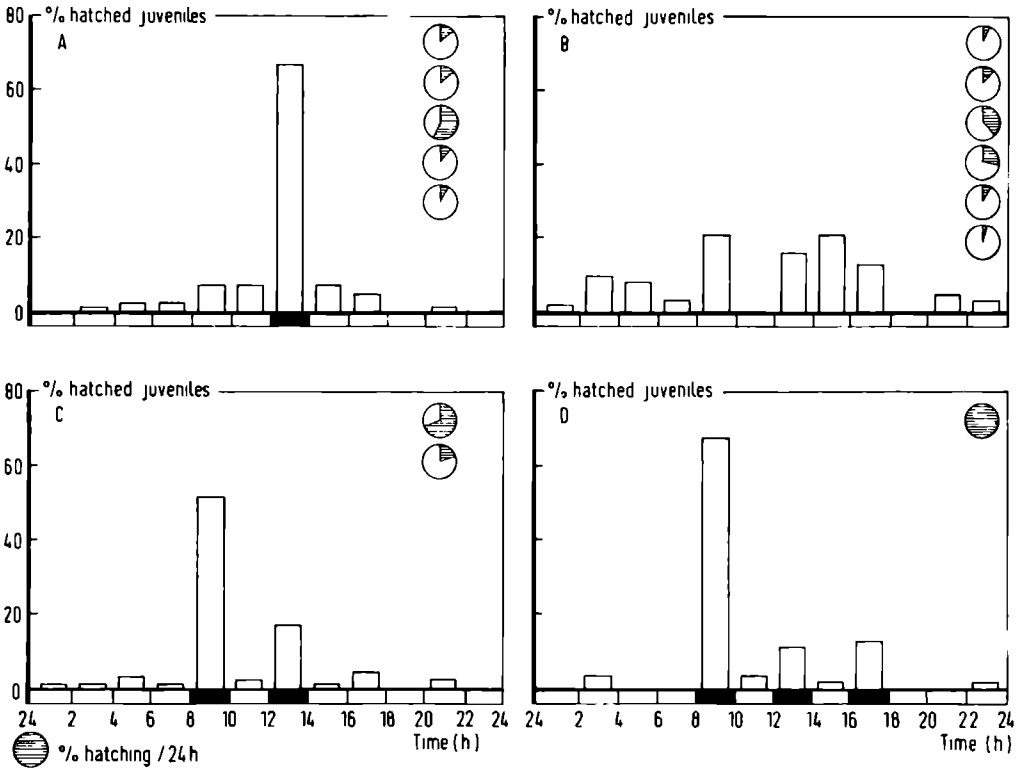

Fig. 8.2. The effect of LD changes at the end of embryonic development of *Loligo vulgaris*. Horizontal bar: white=light period, black=dark period. The embryos in Group 2:1 (A)(control) remained in a LD cycle of 22:2. In Group 2:2 (B) the dark period was eliminated as soon as the majority of the embryos reached stage XX (Naef). In Group 2:3 (C) one and Group 2:4 (D) two extra dark period(s) were added. Cycles = percentage hatching in each of the 24 hr cycles during which hatching occurred. Total number of cycles indicates the total duration of hatching.

In Group 2.2, a total of 63 embryos hatched during six 24 hrs cycles. The null hypothesis, that hatching is equal in all 2 hr periods, was rejected ($p<0.005$). We found higher numbers than expected between 10.00 and 12.00 hr (N=13) and 14.00 and 16.00 hr (N=23). During all other 2 hr periods the amount of hatching per unit of time was less than 10.5 embryos. No hatching took place between 12.00 and 14.00 hr.

Also in Group 2:3 (Fig. 8.2C) significantly higher hatching percentages were found during the two dark periods (G/q test H_o: equal probability of hatching in dark and light periods, $p<0.005$). The total number of hatching during the first dark period, which was introduced as soon as the embryos reached stage XX, differed significantly from the second dark period ($p<0.0005$). Hatching was spread over two 24 hrs cycles, being three periods less than in Group 2:1. During these two 24 hrs cycles the embryos of Group 2:3 received four dark periods. The embryos in Group 2·1 were placed in darkness only twice during two 24 hrs cycles. The hatching rhythm in Group 2:3 differed significantly from Group 2:1 ($p<0.005$).

In Group 2·4 two extra dark periods were introduced as soon as the embryos reached stage XX, *i.e.* light was switched off three times within 24 hrs. In total 64 embryos hatched, 58 of them during darkness. Hatching was spread over one 24 hr period. Numbers hatching during the first dark period differed significantly from the second and the third ($p<0.0005$). During the first dark

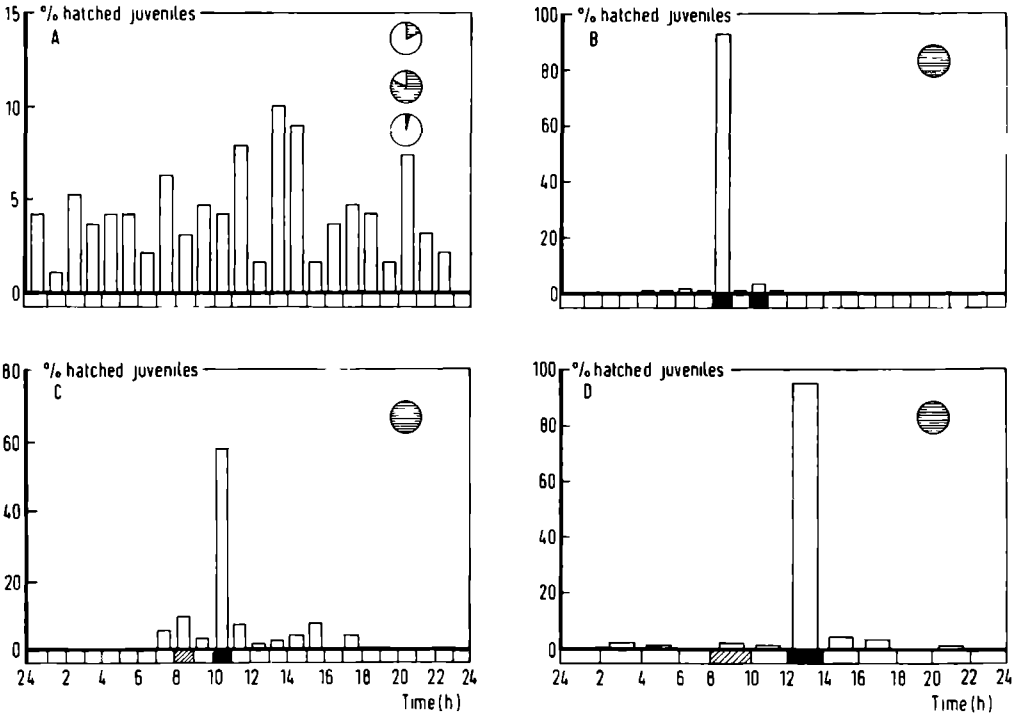

Fig. 8.3. The effect of changes in a constant light regime on hatching of *Loligo vulgaris* embryos. Horizontal bar: white=light period (100 μE s^{-1} m^{-2}), striped=twilight period (50 μE s^{-1} m^{-2}), black=dark period (0 μE s^{-1} m^{-2}). In Group 3:1 (A) (control) the embryos remained in constant light. In all other groups the embryos developed in constant light but as soon as the majority of the embryos reached stage XX (Naef) they were exposed to several light, twilight and dark shocks. Group 3:5 (B) repeated one hour dark shocks starting at 08.00 hr. Group 3·6 (C) twilight shock from 08.00 till 09.00 hr, darkness from 10.00 till 11.00 hr. Group 3:7 (D) twilight from 08.00 till 10.00 hr and darkness from 12.00 till 14.00 hr. Cycles = percentage hatching in each of the 24 hr cycles during which hatching occurred. The total number of cycles indicates the total duration of hatching.

period, from 08.00 till 10.00 hr, 43 embryos hatched while during the second and the third period only seven and eight embryos hatched, respectively There was no significant difference between the second and the third period ($p>0.05$). Hatching during every light period differed significantly from every dark period ($p<0.0005$). There was also a significant difference between hatching of Group 2.1 (control) and 2:4 ($p<0.0005$).

In experiment 3 (Figs 8.3 and 8.4), Group 3·1 serves as the control (Fig. 8.3A): the *L.vulgaris* embryos developed and hatched in continuous illumination (comparable also with Group 1·5 and 1:6). In total 190 juveniles were counted. Hatching was spread over three 24 hr cycles. All juveniles counted were compiled within 1 hr periods. Under the null hypothesis of constant hatching, 15.83 embryos would have been expected to hatch every two hours in Group 3:1. This hypothesis differed significantly ($p<0.025$) from hatching in this group.

In Fig. 8.4 the results of Group 3·2 are shown. Hatching in this group differed significantly from expectation under the null hypothesis of constant hatching ($p<0.0005$). Almost all embryos hatched during the first four-hour dark shock (94.77%) and hatching was spread over one 24 hrs period. Similar results were found in Groups 3:3 and 3 4 (Fig. 8.4). Hatching also differed significantly from expectation under the null hypothesis of constant hatching ($p<0$ 0005) Most embryos hatched during darkness; 89.66% and 94.56% in Groups 3:3 and 3:4 respectively. Although the dark period in Group 3 4 was very short, from 08.00-till 08.10 hr, most embryos hatched during this period. Between Groups 3 2, 3:3 and 3:4 no significant differences were found (RxC test of independence using G-Test: $p>0.05$). In Group 3·5 (Fig. 8 3B) embryos were placed in darkness every other hour as soon as the majority reached stage XX, starting at 08.00 hr Most embryos hatched during the first dark period (92.81%) as in Groups 3:2 to 3:4. A highly significant difference was found between the first and the second dark period ($p<0.0005$). During the second dark period only five embryos (*i.e.* 2.99%) hatched. All juveniles hatched within 8 hrs. Only a few embryos hatched during light and a significant difference was found between the light and dark periods ($p<0.0005$).

All embryos in experiment 3 developed in light with an intensity of 100 $\mu E\ s^{-1}\ m^{-2}$. In Groups 3:6 and 3:7 (Figs. 8.3C and D) the embryos were also exposed to a twilight period, *i.e.* a light reduction of 50%, for 1 or 2 hrs respectively, as soon as the majority of the embryos reached stage XX. No significant difference was found between hatching during light and twilight ($p>0.05$). After the twilight period the embryos were placed in light again for 1 hr (Group 3 6) or 2 hrs (Group 3:7) and following this light period they were put in complete darkness for the same time. After this dark

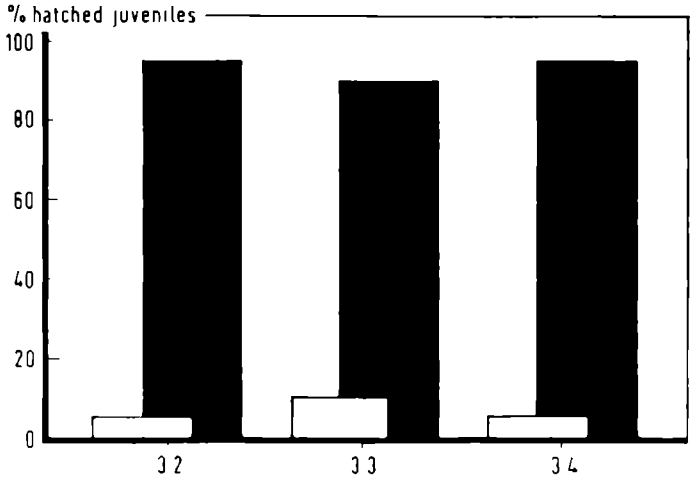

Fig. 8.4. Effect of one dark shock at the end of embryonic development of *Loligo vulgaris* embryos. White bar = light period; black bar = dark period Group 3·2 dark shock from 08 00 till 12.00. Group 3·3 dark shock from 10.00 till 11.00 hr. Group 3·4 dark shock from 8.00 till 8.10.

period most embryos had hatched in both Groups 3:6 and 3:7. Significant differences were found between the twilight or light period and the number of hatching after darkness. In Group 3:7 hatching was spread over 20 hrs and in Group 3:6 over 11 hrs.

In experiment 4 the hatching rhythm during light and darkness of the 12:12 LD cycle was investigated (Fig. 8.5). The way the juveniles were counted had no effect on the hatching rhythm. The embryos which had developed in constant illumination showed a hatching rhythm similar to Group 1:6 and the hatching percentage did not increase directly after the treatment. The embryos which had developed in a 12:12 LD cycle hatched almost exclusively during darkness. From the results it is clear that most embryos hatched shortly after the transition from light to darkness. The lowest hatching percentages were found between 24.00 and 02 00 hr. Hatching during the dark period was not confined to only one 2 hr period.

In Fig. 8.6A four groups of *L. forbesi* eggs from one string are shown, photographed one minute before darkness. Fig. 8.6B was made 15 minutes after darkness. The juveniles hatched between 0 and 15 minutes after the transition from light to dark.

DISCUSSION

Our results indicate that LD periods exert a profound effect on the timing of hatching in the squids *L.vulgaris* and *L.forbesi*. Most embryos hatch shortly after the onset of darkness. The transition from light to dark acts as a "Zeitgeber" or synchronizer. The function of a synchronizer is to inform the organism about the period and phase of the universal time (Sollberger, 1965). A different situation is found in fish: embryos of the medaka (*Oryzias latipes*) and of the zebrafish (*Brachydanio rerio*), which had been kept in a 12:12 LD cycle, showed a significantly higher hatching rate in the light period than in the dark period (Schoots et al., 1983b). Exposure to constant darkness, from fertilization to hatching, strongly suppressed hatching of medaka embryos (Yamagami and Hamazaki, 1985).

In our experiments hatching was not stimulated if the light intensity was reduced by 50%. To stimulate squid hatching the threshold value probably must be less than 50 $\mu E\ s^{-1}\ m^{-2}$ and/or hatching can only be stimulated if the light is reduced by more than 50%.

Temperature may probably also synchronize hatching of *L.forbesi* and *L.vulgaris*. This is indicated in experiment 1. In Groups 1·2 and 1:3 a few embryos hatched during light but in Group 1:1, in which the temperature fluctuated between day and night, no hatching occurred during the light period at all. It is conceivable that the weak synchronizing effect of temperature becomes masked

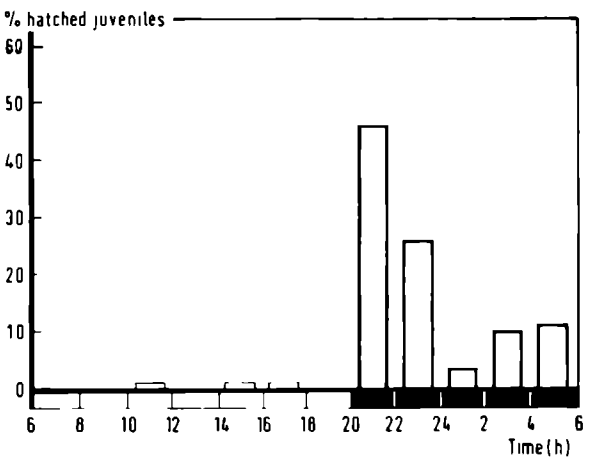

Fig. 8 5. Percentage *Loligo vulgaris* juveniles, hatched during intervals of two hours within a 12·12 LD cycle. Horizontal bar. white=light period, black=dark period.

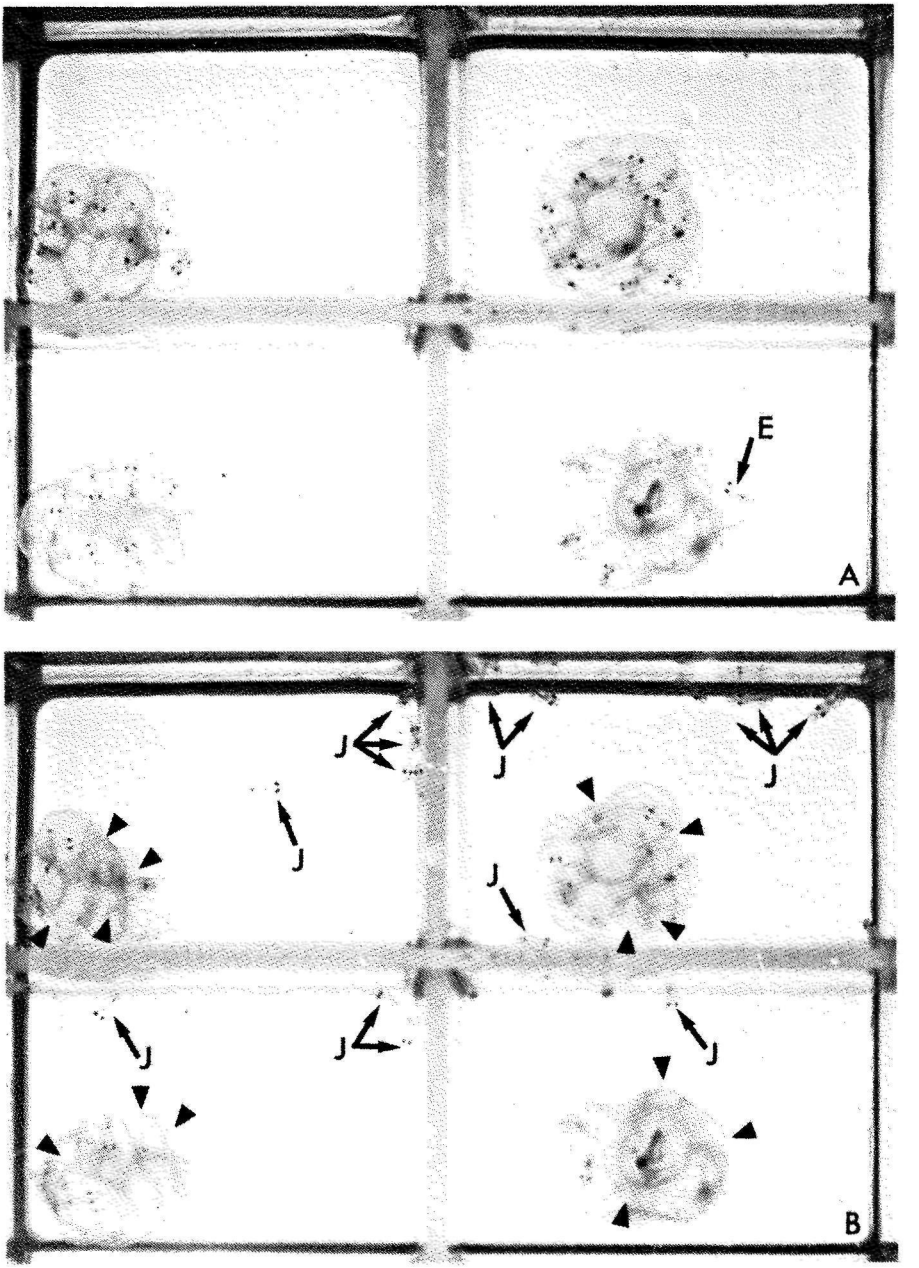

Fig. 8.6. Hatching registration of embryos of *Loligo forbesi*. One strand of eggs is divided into 4 pieces. The outer jelly coat is removed. The first picture (A) was made one minute before darkness. E = embryo. Fig. 8.6B shows the strand pieces and juveniles (J) 15 minutes after the onset of darkness. The black triangles indicate empty chorions.

by dominant synchronizers, such as light, as soon as they appear.

Individuals reared under constant illumination conditions, as in Groups 1:5 and 1:6, did not exhibit any hatching rhythm at all. Before being used for the experiments, these embryos were placed in an outside aquarium and received natural light. The results of experiment 1 and 3 indicate that this natural LD rhythm (and probably any given rhythm) is no longer conserved after stage X So, if any endogenous hatching rhythm exists, this can only be set in motion or be entrained after stage X (Naef 1923; 1928).

The embryos in Group 2:2 (dark period was eliminated as soon as the embryos reached stage XX) showed a hatching rhythm which differed significantly from Group 2:1 in which the 22 2 LD cycle was maintained from stage X on. In Group 2.2 hatching occurred around the time the dark period was expected. These results do not prove the existence of any endogenous rhythm but there is clearly a preference for a period which coincides with the period at which darkness usually occurred In continuous light, the rhythmicity probably damps out very quickly.

Sometimes a single disturbance *e.g.* a dark-shock, can set an endogenous rhythm in motion (Sollberger, 1965). The results of experiment 3 however, could not indicate the existence of any rhythm because the dark-shock had such a tremendous effect on hatching that most embryos hatched during the (first) dark period Hatching was confined to just one 24 hr cycle.

If any external LD rhythm was present, embryos preferred to hatch during darkness, even when the duration of the dark period was very short (compare with Groups 1 4 and 2:1) or shifted to another time (compare with experiment 2). In these cases hatching was completely driven by the external LD rhythm. Our results also indicated that during artificial LD cycles of about the same time and length as in natural environments (compare with experiment 4) hatching was not limited to one particular period during the night, although most embryos hatched shortly after darkness. Investigations made by Laroe (1971) on embryos of *Sepioteuthis sepioidea* Blainville, 1823, and *Doryteuthis plei* Blainville, 1823, indicated that this is not the same for every cephalopod species. *S.sepioidea* and *D.plei* embryos also prefer to hatch during darkness but most hatched primarily late at night or during early twilight, between 01.00 and 07.00 hr. If the laboratory lights were kept dim, a high number continued to emerge until 08.00 hr.

Hatching is synchronized only if light and dark transitions can be detected by the embryos. In our experiments the egg strands were hanging about 7-15 cm deep in the sea water. At this shallow depth, almost 100% of light of a rather long wavelength can still be detected (Poole and Atkins, 1937). In natural circumstances *Loligo* eggs are also generally deposited in relatively shallow water (Worms, 1983). In the Oosterschelde, *L.vulgaris* spawns are found at depths between 1 and 21 m, but frequently between 3 and 6 m (own observations). Eggs are often attached to fishing nets The water in the Oosterschelde is rather clear so there is little attenuation of light. In 1989, during spring and summer, 1% of the natural light could still be detected at an average depth of 7.47 m. Also on average 100 $\mu E\ s^{-1}\ m^{-2}$ could still be detected at 1.55 m (P. de Visscher, DIHO, pers. comm.). Probably, in some particular natural conditions light and dark transitions will synchronize hatching of *L.vulgaris* and *L.forbesi*.

At this moment our results cannot give any indication about the photosensory system which is responsible for the perception of the light-dark rhythm in *L vulgaris* and *L.forbesi*. Of all the invertebrate sensory organs, the cephalopod eye has probably received most attention (Arnold, 1971). In recent years evidence has been accumulating in support of the view that cephalopods have other photoreceptors besides the eye. In decapod cephalopods, so-called parolfactory vesicles are found near the optic tract (Boycott and Young, 1956). It has been shown that these vesicles contain considerable amounts of rhodopsin. This had led to speculations that the vesicles act as photoreceptor organs (Nishioka et al., 1966). That these extraocular receptors in cephalopods act as light detectors is indicated by the absence of a lens apparatus and of any oriented array of radially extending rhabdomeric processes to form a "retina", as in the eye proper (Mauro, 1977). In *L.vulgaris* stage XX embryos these vesicles are very small and hardly developed (Prof. J.Z. Young, pers. comm). In 1982 Wittland and Fioroni described in *L.vulgaris* and in some other species of dibranchiate cephalopods, three pairs of ectodermal vesicles. The function of these vesicles is probably also photoreceptive. This assumption is supported by a study of Von Boletzky et al. (1970).

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THE INFLUENCE OF PHOTOPERIODICITY ON HATCHING OF *SEPIA OFFICINALIS* RELATED TO SOME PROPERTIES OF THE EGG CAPSULE DURING EMBRYONIC DEVELOPMENT*

ABSTRACT. The influence of photoperiodicity on hatching of *Sepia officinalis* was investigated under different experimental light-dark (LD) conditions For an interpretation of the results some properties of the perivitelline fluid (PVF) and the egg capsule during embryonic development were taken into consideration. In embryos of *S.officinalis* the transition from light to dark acts as a "Zeitgeber" or synchronizer. The embryos preferred to hatch during darkness, even when the dark period was short (1-4 hrs) and replaced part of the natural light period. The hatching rhythm was also independent of the embryonic stage in which the experiment was started. LD rhythmicity was never conserved. In the absence of any external LD rhythm the time-to-hatching increased Lack of pigmentation in the egg envelope strongly decreased the time-to-hatching When embryos were exposed to a single dark period of 1 to 4 hrs, many of them hatched during darkness. On the contrary, during a dark shock of 10 minutes hatching never took place. When embryonic development is near completion the egg capsule of *S.officinalis* becomes thinner as a result of the expanding PVF. Absence of the envelope did not affect embryonic development. In *Sepia* the envelope has mainly a consolidating function It compensates the osmotic pressure of the PVF Spectrophotometrical investigations indicated that light between 200 and 900 nm is absorbed by the envelope and by ink from a female adult. The role of the black pigment in the envelopes remains obscure.

INTRODUCTION

According to recent investigations of Pauli et al. (Chapter 8 of this thesis) hatching of *Loligo vulgaris* Lamarck, 1798 (European squid) and *Loligo forbesi* Steenstrup, 1856 (veined squid) is affected by various light conditions. In both species the transition from light to dark stimulates hatching and functions as a "Zeitgeber" or synchronizer In most natural environments, depending on the clearness of the water, light can reach the developing *Loligo* embryos because the eggs are deposited generally in relatively shallow water and have quite translucent egg capsules (Fioroni, 1978; Worms, 1983)

A different situation is found in the species *Sepia officinalis* Linnaeus, 1758 (common cuttlefish) In contrast to the egg capsules of *Loligo* the spirally coiled egg envelopes of *S officinalis* are normally

* Chapter 9 is the modified version of Pauli, W P , Herman, P M J Roozen, M E F , Denucé, J M (submitted) The influence of photoperiodicity on hatching of *Sepia officinalis* related to some properties of the egg capsule during embryonic development

stained with black ink consisting mainly of sepiomelanin (Lemaire, 1971). In some cases, because of (temporary) lack of ink production in the spawning female, a few eggs within a spawn are not (completely) stained and remain transparent. Embryonic development is not disturbed by this lack of pigmentation (Lemaire, 1971; Von Boletzky, 1983). Like *L.vulgaris* spawns, cuttlefish eggs are often found in the Oosterschelde, part of the Delta Area (South Western part of The Netherlands) (Chapter 10). The shallow water of the Oosterschelde is rather clear so there is little attenuation of light (Chapter 8).

During embryonic development of *S.officinalis* the elastic envelope is dilated by the chorion which expands due to the osmotic pressure of the perivitelline fluid (PVF). PVF is slightly hypertonic to sea water (De Leersnyder and Lemaire, 1972). The envelope reaches a maximum diameter of about 1.5 cm by the time of hatching (Fioroni, 1978; Von Boletzky, 1983). Due to the expanding PVF, the envelope and the chorion become rather thin at the end of embryonic development (Lemaire, 1971). Therefore, in late embryonic stages not only the protection by the egg capsule declines (Chapter 7) but also environmental factors such as light can more easily affect the developing embryo.

In this chapter the effect of light on hatching of *S.officinalis* was investigated. During the investigations we used black, partly stained (grey) and translucent eggs. The results of the experimental study are compared with data concerning some characteristics of the PVF and the egg capsule during embryonic development.

MATERIAL AND METHODS

Experimental study

The study was carried out at the Delta Institute for Hydrobiological Research (DIHO) in Yerseke, The Netherlands, and at the Catholic University in Nijmegen (Department of Zoology I), The Netherlands, between 1988 and 1990, with embryos of *Sepia officinalis*. The eggs were collected by fishermen from the Oosterschelde during spring and summer. In spring 1988 and 1989 spawns

Table 9.I. Schedule of experiment 1.

Group	N	LD cycle	time of dark period(h)	colour egg capsule	start experiment
1:1	121	7:17	17.00-10.00	black	24 h after spawning
1:2	33	7:17	17.00-10.00	grey	24 h after spawning
1:3	19	7:17	17.00-10.00	translucent	24 h after spawning
1:4	35	constant illumination		black	24 h after spawning
1:5	79	14:10	20.00-06.00	black	stage 21-24
1:6	55	constant illumination		black	stage 21-24
1:7	40	17.5-6.5	16.00-09.30	black	stage 21-24
1:8	21	constant illumination		black	stage 21-24
1:9	88	20:4	10.00-14.00	black	stage 26-28
1:10	85	23:1	10.00-11.00	black	stage 26-28

Table 9.II. Schedule of experiment 2

Group	N	start time dark-shock	duration of dark-shock
2·1	27	14.30	10 min
2:2	92	18 55	10 min
2 3	82	16 00	1 h
2:4	36	09.20	1 h
2.5	51	14 45	1 h
2·6	37	09.20	2 h
2 7	57	11.40	3 h
2·8	51	16.45	3 h
2:9	55	10.25	4 h
2.10	100	11.00	4 h

were also obtained from sexually mature specimens kept in sea water tanks (Chapters 7 and 10).

To ensure good conditions of development, the eggs from a bunch were separated and were laid on the bottom of a perforated plastic box suspended in an aquarium (capacity 50 litres), without mixing the eggs. All aquaria were provided with running sea water coming directly from the Oosterschelde.

The staging and numbering system of Lemaire (1970) (Stage 30 = hatching stage) was applied to indicate the age of the embryos. The embryos received natural light until they were used for the various experiments.

All experiments were performed in perspex aquaria containing 12 litres of filtered Oosterschelde water (capacity 20 litres) and were equipped with specially constructed floats made of 12 perforated plastic tubes (Chapter 10). Each aquarium was connected to a separate pumping device which enabled a closed circulation system without the need for extra aeration.

Experiment 1 was carried out in automatically controlled climate chambers kept at a constant temperature of 16°C. The temperature of the water and the air was continuously monitored to ensure that the light did not heat up the water The light intensity, measured with a Metrawatt meter (model Tavolux 2 PL X) with a platinum opal filter was 60 $\mu E\ s^{-1}\ m^{-2}$ in light and 0 $\mu E\ s^{-1}\ m^{-2}$ in dark periods

Experiment 1 is summarized in Table 9.I. Switchings between light and darkness were abrupt, without gradual transition. To determine the developmental stage of the embryos at the beginning of the experiment, 50 eggs from each spawn were taken at random and the embryos obtained were staged. The remaining eggs were used for the various groups.

For Groups 1:1 to 1·4 altogether two batches were used 24 hrs after spawning. They contained a large number of eggs with grey or translucent envelopes (Fig. 9 1) These eggs were used for Groups 1:2 and 1·3 In all other groups and experiments only embryos surrounded by black egg capsules were used

In the remaining groups of experiment 1 in total three spawns were used: the first for Groups 1.5 and 1:6, the second for 1:7 and 1:8 and the third for 1·9 and 1:10. Juveniles were counted a few minutes before and after the dark period or the light period (Groups 1:4, 1:6 and 1:8) and were

removed immediately from the aquaria without disturbing the remaining embryos. After all embryos had hatched (or died) the hatching percentage in each period was calculated ([hatching percentage = number juveniles : total number of juveniles] x 100).

All the embryos used for experiment 2 developed in natural light conditions until they reached stage 30. The experiment was carried out in August 1990. During the experimental period sunset occurred around 6.00 h and darkness fell at about 21.00 h. Experiment 2 is summarized in Table 9.II. As soon as the first embryos from one spawn had hatched, the remaining eggs were very gently transferred to plastic aquaria, filled with 7 litres Oosterschelde water taken from the aquarium in which the embryos had developed till stage 30. The embryos were left undisturbed for at least 2 hours before being placed in darkness. The temperature of the water was controlled before and after the experiment. To ensure that this treatment had no effect on the hatching rhythm, altogether 159 embryos were treated the same way, except that they remained continuously exposed to natural light. After the dark-shock the juveniles were counted and the hatching percentage was calculated as described above.

In experiment 1 G/q single classification goodness-of-fit tests (Sokal and Rohlf, 1981, p. 705) were used to test if hatching was distributed homogeneously over different time periods. As a null hypothesis (external to the sampled data) it was assumed that the proportion of embryos hatching in one period was equal to the proportion of that period to the whole day. Thus, when n embryos hatched in a L:D rhythm of 14:10 hr, the null hypothesis was that n*14/24 embryos hatched in the light period, and n*10/24 embryos in the dark period. The test was two-sided.

An RxC test of independence using G/q (Sokal and Rohlf, 1981, p. 745) was used to test homogeneity of different data sets. The effect of pigmentation and light on the timing of hatching was tested by comparing simultaneous comparison intervals around the medians, as visualised in box and whisker plots (McGill et al., 1978; Wilkinson, 1988). Non-overlapping intervals indicate a 95% probability that the population medians are different.

In experiment 2 the probability of hatching was regressed on length of the dark period, length of the preceding light period and interaction between the two factors. For this analysis logit regression was used (Steinberg, 1985).

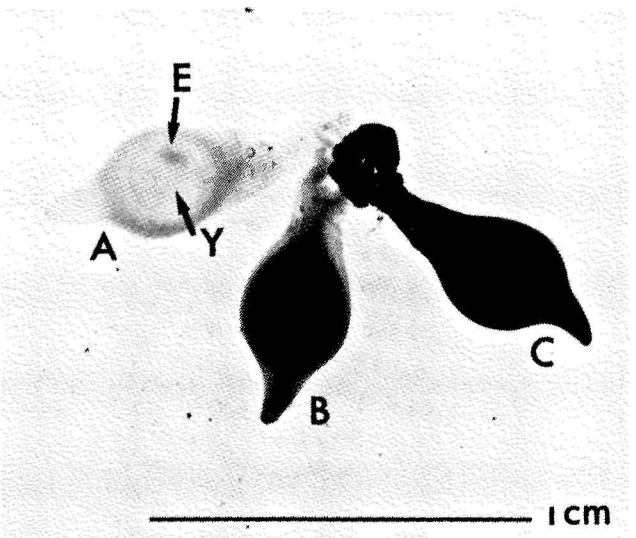

Fig. 9.1. Eggs of *Sepia officinalis* in developmental stage 22 (Lemaire, 1970). A: Egg covered by almost translucent, unstained capsule. The embryo (E) is visible. Y=external yolk sac. B: Egg surrounded by a partly stained (grey) capsule. Only the inner envelope layers are stained black with ink. The outer envelopes are translucent. C: Egg covered by black capsule (normal case).

Visualisation of hatching
To visualize the effect of dark-shocks on hatching of *S.officinalis*, specially devised time-lapse photographic equipment was placed in a separate climate chamber (Chapter 8). Embryos with black capsules, which had developed till stage 30 in continuous illumination, were placed in a cuvette and photographed immediately before and after a one hour dark-shock. In total 25 control embryos were treated as above but remained in constant illumination.

Removal of the envelope
From 50 eggs in stage 20 the envelopes were removed as follows: the tip of the egg was removed using small scissors, and the chorion, the embryo and the PVF was pinched out the hole thus produced. Eggs with intact chorions were replaced in the aquarium in the controlled climate chambers The embryos either received continuous illumination, or they were exposed to the same LD cycle as Group 1:5. Development was checked on a daily basis As soon as stage 28 was reached the eggs were taken out of the aquarium and placed in cuvettes as described above. The water was renewed twice a day The embryos were photographed before, during and after hatching or just before and after the dark period. To make sure that this treatment did not affect the hatching rhythm, in total 25 embryos were observed in the aquarium. A total of 50 black, untreated eggs from the same batch served as a second control.

Light microscopy
Black coloured egg capsules from embryos in stage 22 and 30 and from 1 hour old juveniles were fixed in Hollande's fixative (Gurr, 1962). The fixed material was dehydrated and embedded in paraffin according to conventional histological procedures Sections (5 µm) were deparaffinized and stained with Haemalum-Eosin (HE) The sections were examined and photographed using a 100 ASA Kodak film.

Collection of perivitelline fluid (PVF)
The PVF of *S officinalis* embryos in various stages of development was collected (N=50). After removal from the aquaria the eggs were placed in a dry petridish, cooled with melting ice. The egg capsules were broken with two watchmaker forceps. The morphology, condition and stage of the embryo were checked. If the embryo had developed normally, the PVF was collected with a Gilson pipette From each egg the total volume of PVF was measured.

Spectrophotometry
The absorbance spectrum of black stained envelopes of stage 30 embryos and ink from the ink sac of a mature female was investigated using a Beckman DU 40 spectrophotometer. The envelope was separated from the chorion with two dissection needles and rinsed three times for 5 minutes in distilled water. A small piece of the envelope was stretched over the outer wall of a quartz cuvette and dried at room temperature The ink was diluted with distilled water. Spectral characteristics were measured between 200 and 900 nm.

RESULTS

Experimental study
The results of experiment 1 are given in Fig. 9.2. In Groups 1:1 to 1:3, 1:5, 1:7, 1·9 and 1:10 the frequency of hatching during light periods differed significantly from hatching during darkness (two-sided G/q test; H_0: number of hatching events in light and dark periods is proportional to the relative length of these periods; $p<0.0005$, Group 1 3 = $p<0.005$). The highest hatching percentages were found during darkness, although in most groups some hatching also occurred during light.

The eggs in Groups 1:1 to 1:3 (Figs. 9.2A to C) differed in pigmentation of the envelopes but the hatching rhythm in all three groups was directly related to the external LD cycle. There was no significant difference in hatching rhythm between these groups (RxC test of independence, p>0 05)

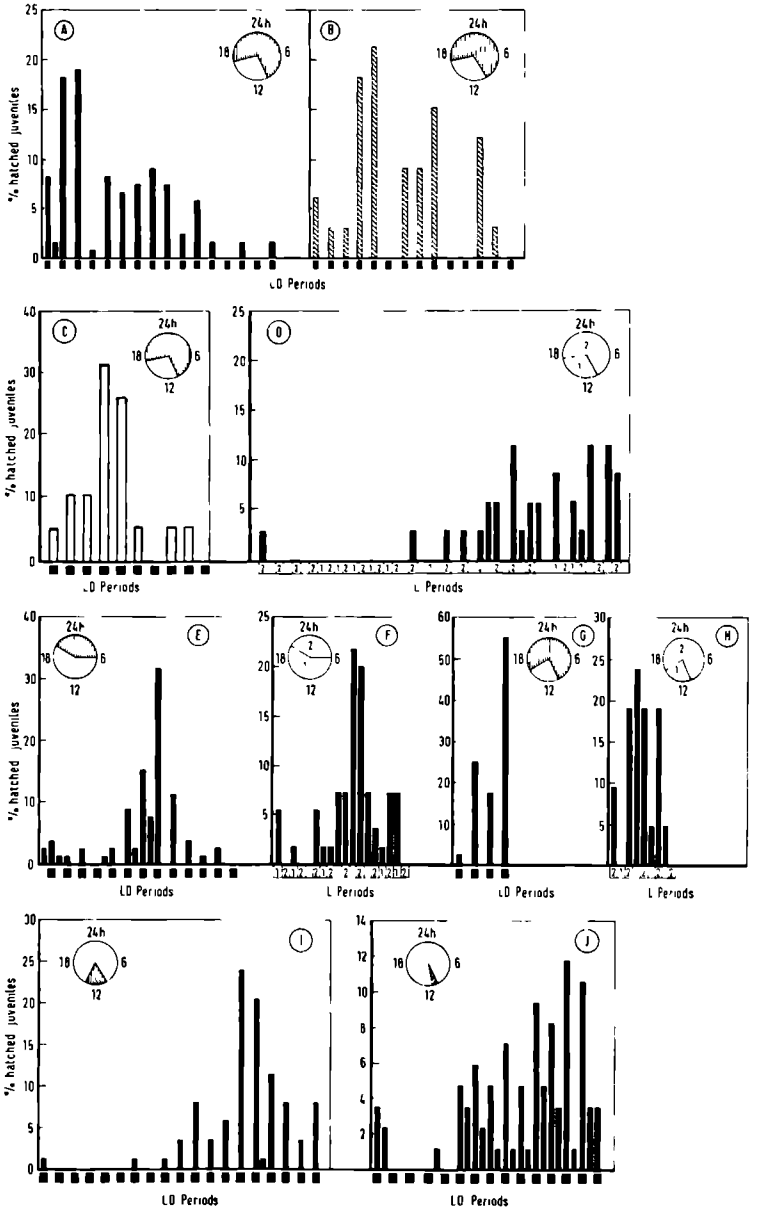

Fig. 9.2. The effect of LD periods and continuous light on the hatching rhythm of *Sepia officinalis*. Horizontal bar: white=light period, black=dark period. Vertical bar: white=translucent egg envelopes, striped=grey or partly stained egg envelopes, black=black egg envelopes. (A)Group 1:1, (B)Group 1:2, (C)Group 1:3, (D)Group 1:4, (E)Group 1:5, (F)Group 1:6, (G)Group 1:7, (H)Group 1:8, (I)Group 1:9, (J)Group 1:10. Cycles: LD rhythm (striped part is dark period) or LL rhythm (numbered periods correspond to numbers in horizontal bar). Temperature was kept constant at 16°C. Light intensity during illumination was 60 μE s^{-1} m^{-2} and during darkness 0 μE s^{-1} m^{-2}.

In Group 1:3 (Fig. 9.2C) all embryos hatched within nine consecutive 24 hr cycles. The embryos in this group developed in eggs with almost translucent capsules In Groups 1:2 (Fig 9.2B)(grey egg capsules) and 1:1 (Fig. 9 2A)(black egg capsules) hatching was spread over 13 and 16 LD cycles, respectively. In Group 1:4 (Fig. 9.2D) the embryos developed within black capsules and were exposed to constant illumination. In this group most embryos hatched within 13 LD cycles while the total amount of hatching was spread over 22 cycles Egg capsule pigmentation and LD rhythm had a significant effect on median duration of embryonic development in these groups Time-to-hatching increased in the absence of an external LD rhythm (group 1 4) If the LD rhythm was present, lack of pigmentation in the egg capsule lowered time-to-hatching (groups 1:1 to 1.3)

In Group 1:4, 1:6 and 1:8 (Figs. 9.2D, F and H) embryos developed in constant illumination In Fig. 9.2 the hatching rhythm of these groups, related to the LD cycles of the corresponding groups, is given. The number of embryos hatched in the two periods of the 24 h cycle that correspond to the former L and D periods, was proportional to the lengths of these periods (G/q test H_0: hatching is proportional to relative length of the periods, $p>0.05$). The embryos hatched as soon as their development was accomplished, completely independent of any period within the 24 hr cycle and the embryonic stage in which the experiment was started. Apparently, the natural LD rhythm to which the embryos were exposed before the experiment was started was not preserved.

In Groups 1:9 and 1:10 (Figs. 9.2I and J) embryos were exposed to natural light conditions before they reached stage 26-28 From stage 26-28 on the embryos were placed in a climate chamber and a short dark period was introduced from 10 00 hr on. Also in these groups hatching was completely related to the external artificial LD cycle, despite the short duration and the timing of the dark period, and the advanced embryonic stages in which the experiment started Most hatching occurred during darkness but in Group 1:10 (Fig. 9.2J) in which the embryos were exposed to a one hour dark period, a higher number of embryos emerged during light than in Group 1.9 (Fig 9.2I), in which the dark period lasted for four hours.

The embryos in experiment 2 were exposed to a single dark-shock at a time light normally occurred. Before the embryos had reached stage 30 (*i e* hatching stage) they developed in a natural LD cycle. The results of experiment 2 are given in Fig 9.3. In Groups 2·3 to 2 10 the dark-shock initiated hatching and many embryos hatched during darkness In these groups the duration of darkness varied between 1 and 4 hrs. No hatching was observed if the embryos were exposed to a dark-shock of 10 minutes (Groups 2·1 and 2:2). Using the logit regression test a highly

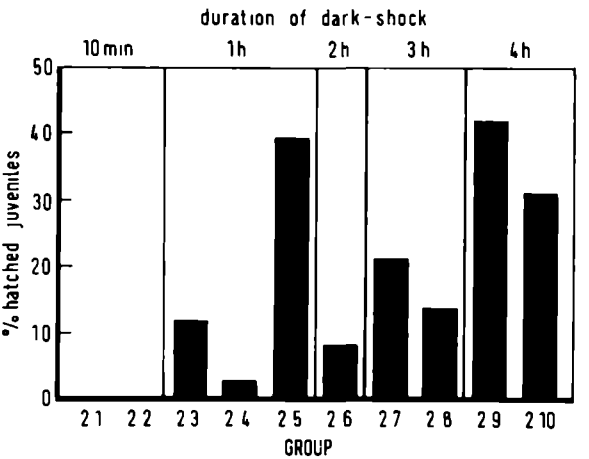

Fig. 9 3 Effect of a single dark-shock at the end of embryonic development on hatching of *Sepia officinalis* embryos.

significant relation was found between the probability of hatching and the duration of the dark-shock. More embryos hatched when the dark period was extended. The timing of the dark-shock within the natural 24 hr LD cycle, *i.e.* the length of the light period before the experiment started, had no significant effect on the amount of hatching during the dark-shock nor was there a significant effect of the interaction between length of dark period and length of preceding light period.

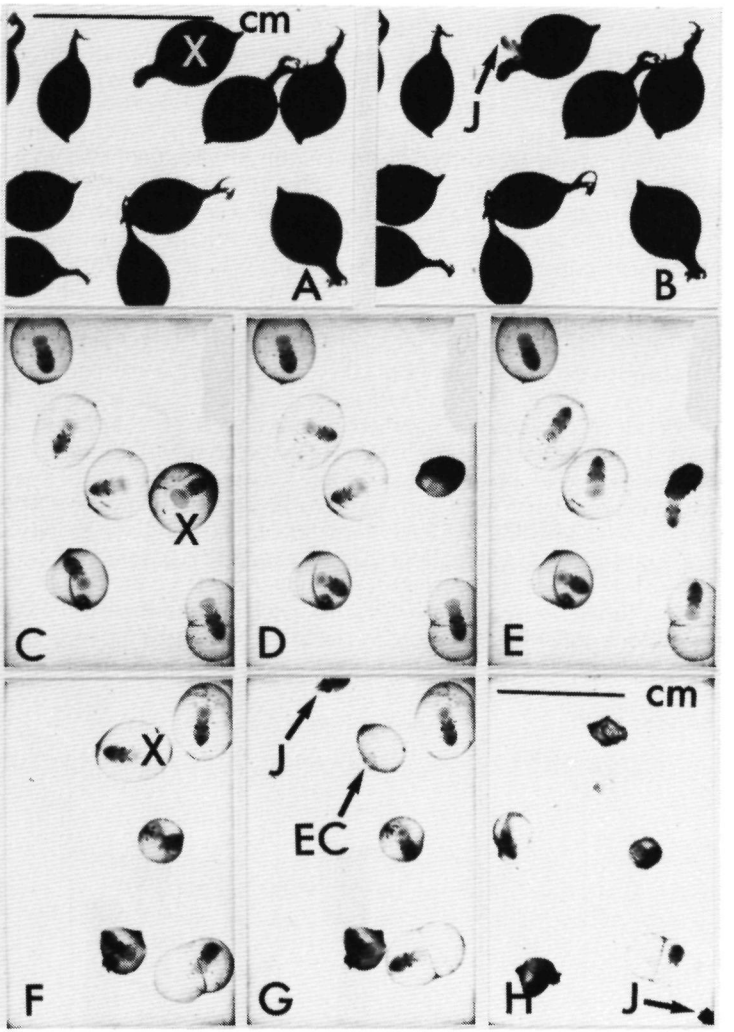

Fig. 9.4. Hatching registration of *Sepia officinalis*. Normal black stained eggs which were kept in continuous illumination, photographed just before (4A) and after (4B) a one hour dark-shock. X=egg before hatching; J=juvenile. Figs. 9.4C to H: eggs without envelopes. Figs. 9.4C,D and E: during embryonic development the chorion ruptured due to the expanding PVF. As a result the chorion starts to shrivel up (4D) and the embryo (X) hatched prematurely (4E). Figs. 9.4F,G and H: "normal hatching". The embryo (X) hatches by locally dissolving the chorion and immediately after hatching the external yolk sac is thrown off (4G). The empty chorion (EC) shrivels up after hatching (4H). J=juvenile.

In Fig. 9.4A *S.officinalis* eggs from one batch are shown, photographed one minute before a one hour dark-shock. The embryos had developed under continuous illumination. Fig. 9.4B was made directly after the one hour dark period. The juvenile had hatched during darkness.

Removal of the envelope

The absence of the envelope had no effect on embryonic development. All embryos developed normally and showed no external morphological malformations. But removal of the envelope did affect the survival percentage. From the embryos developing in eggs without envelopes (EWE) 24% died before they had reached stage 28. In total 6% of the control embryos died before hatching. In both controls and EWE the volume of the PVF increased during embryonic development but at the time of hatching the total amount in the EWE was considerabely higher. Therefore, at the end of embryonic development the EWE became larger than the untreated eggs.

In many EWE the chorion ruptured before the embryo had reached stage 30. When this happened the embryo always tried to hatch (Figs. 9.4C, D and E) independently of the external LD conditions. In most cases the embryos developing in the EWE were able to hatch but the absence

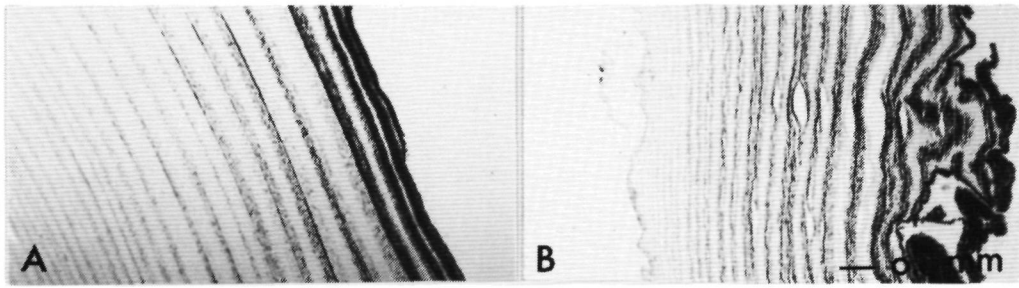

Fig. 9.5. Light microscopic view of the envelope of black stained *Sepia officinalis* eggs. A: stage 22 (Lemaire), B: stage 30 (hatching stage).

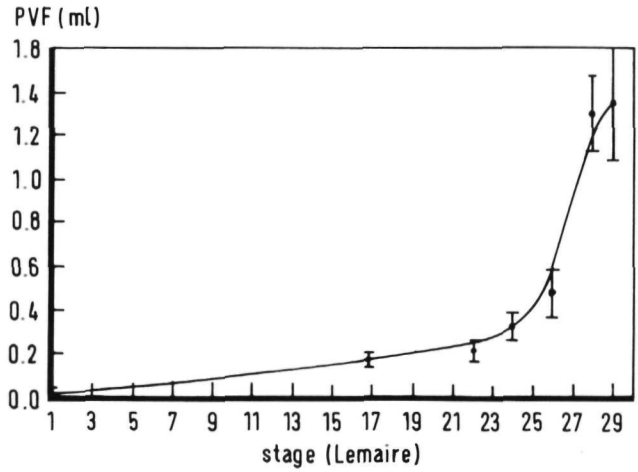

Fig. 9.6. Amount of perivitelline fluid (PVF) of *Sepia officinalis* in various developmental stages according to the staging and numbering system of Lemaire (1970).

of the envelope affected the timing of hatching. Compared to the controls, developing at exactly the same temperature (16°C), the embryos from the EWE started to hatch 7 days earlier. Most of these embryos hatched prematurely (96%). During hatching the embryos still possesed a rather large external yolk sac (Figs. 9.4F, G and J). Most of the premature juveniles died within 24 hrs after hatching. Premature hatching was never observed in untreated eggs.

If the chorion remained intact until the embryos started to hatch, the absence of the envelope had no effect on the hatching rhythm. Embryos from EWE developing in a LD cycle hatched during the dark period. The embryos developing in constant illumination showed no hatching rhythm at all. The same observations were made for the control embryos developing in untreated eggs.

Light microscopy

The envelope of black coloured eggs consisted of several compact concentric layers (Fig. 9.5A). Part of each layer was stained black with ink The total number of layers varied between 20 and 34. During late embryonic development, from stage 26 onwards, the thickness of the egg envelope decreased considerably but the total number of layers did not change. After stage 26 the envelope became also less compact. In stage 30 many small longitudinal tears were found between and inside the individual layers (Fig. 9.5B).

The morphology of the chorion did not change throughout development. During hatching the chorion was locally digested (cf. Fig. 9.4C). On the spot where the embryo had left the egg capsule the chorion was absent but the remnants showed no visible structural changes. The egg capsules became covered with white, thready fungi within 24 hrs after hatching.

Volume of perivitelline fluid (PVF)

The total volume of PVF collected in various embryonic stages is given in Fig. 9.6. During late

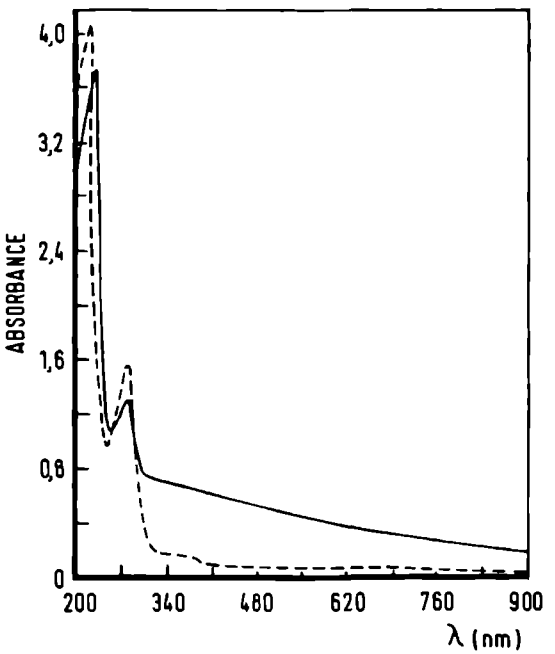

Fig. 9 7 Absorbance characteristics of *Sepia officinalis* ink (- - -) and of black stained egg envelopes (—).

embryonic development the quantity of PVF increased considerably The highest increase was found between stage 26 and 28. In stage 28 the total volume of PVF (1280.00 ± SD 175.36 µl (N=10) was almost three times as high as in stage 26 (456.36 ± SD 65.62 µl (N=11).

Spectrophotometry

Spectrophotometrical investigations showed a strong similarity between the absorbance spectrum of the ink and the black stained egg envelopes (Fig. 9.7). In both cases the highest absorbance was found at 230 and 270 nm. Depending on the ink concentration or the thickness of the envelope also light of other wavelengths was absorbed. Absorbancy always declined gradually towards longer wavelengths.

DISCUSSION

Our experimental study indicated that in embryos of *S.officinalis* the transition from light to darkness acts as a "Zeitgeber" or synchronizer. If any external LD rhythm was present, embryos preferred to hatch during darkness, even when the duration of the dark period was rather short (1 or 4 hrs) and shifted to a time when light normally occurred (compare Groups 1:9 and 1:10) *S.officinalis* embryos reared under constant illumination conditions (Groups 1·4, 1:6 and 1:8) failed to exhibit any hatching rhythm. These results are completely in agreement with observations made with the squids *L.vulgaris* and *L.forbesi* (Chapter 8).

In Groups 1:5 to 1:8 the experiment was started in stages 21-24, *i.e.* about halfway the embryonic development. In Groups 1:9 and 1:10 the experiment began at the end of development, *i.e.* in stages 26-28. Before being used for experiment 1, all embryos received natural light. The results indicated that this natural LD rhythm (and probably any given rhythm) is not conserved. Similar observations were made with *L.vulgaris* and *L.forbesi* embryos (Chapter 8).

The absence of a LD rhythm had a significant effect on the time-to-hatching in *S.officinalis*. Under continuous light conditions the time-to-hatching increased. Hatching was neither stimulated nor synchronized. The embryos emerged from the capsules as soon as their development was completed On the contrary, lack of pigmentation in the envelopes strongly reduced the time-to-hatching within a group. This can be explained by the fact that in translucent eggs the external LD rhythm can more easily be detected by the embryos.

In experiment 2 embryos were exposed to a single dark-shock at the end of embryonic development (hatching stage) at a time light would normally shine. Before the experiment started, the embryos were exposed to a natural LD rhythm If the dark-shock lasted between 1 and 4 hrs, large numbers of embryos hatched during darkness, independent of the timing of the dark-shock. Apparently, a single dark period of 1 to 4 hrs initiates hatching and the hatching process itself takes place during this time But during a dark-shock of 10 minutes no hatching was found. This indicated that in *S.officinalis* a dark-shock of 10 minutes does not initiate the hatching process and/or the initiation of hatching and the hatching process itself takes longer than 10 minutes. The last point mentioned may explain why some embryos hatched during the consecutive light period.

The results of experiment 2 indicated an interesting difference between *Loligo* and *Sepia*. Observations of Paulij et al. (Chapter 8 of this thesis) indicated that in *L.vulgaris* and *L.forbesi* 94.56 % of the embryos hatched during a single dark-shock of 10 minutes. The same results were found when embryos were exposed to longer single dark periods. In squids no relation could be established between the length of the dark period and the total amount of hatching like in *S.officinalis*. Apparently, the hatching process in *L.vulgaris* and *L.forbesi* is faster than in *Sepia* and/or to initiate hatching in *Sepia* a longer dark period is needed.

Our results indicate that at the end of embryonic development even black eggs of *S.officinalis* are capable of detecting light transitions because the hatching rhythm is entirely driven by external LD conditions. This is confirmed by our structural studies of the envelope. During embryonic development *S.officinalis* egg capsules become thinner and less compact, but in disagreement with the investigations of Lemaire (1971) the total number of envelope layers does not decrease. In particular between stages 26 and 28 a strong increase of PVF is found and as a result the egg capsule is stretched and probably becomes more penetrable for light.

The consequences of envelope removal in *S officinalis* indicated that the envelope has an important consolidating function (cf. Chapter 7). After removal of the envelope a large percentage of the embryos died and premature hatching occurred. The chorion often ruptured before hatching. From these observations it can be concluded that the envelope is important for compensating the osmotic pressure produced by the expanding PVF In eggs from which the envelopes had been removed the total amount of PVF became even larger at the time of hatching. As a result the PVF probably became more isotonic to sea water than in untreated eggs, which could have affected the embryonic stage in which the embryos hatched. This explains the high number of premature juveniles in the experimental group.

Our spectrophotometric investigations do not provide any indication about the characteristics of the light which is being detected by the embryo. The absorbance spectrum of the black stained envelope is very similar to the spectral characteristics of ink. Only light of very short wavelengths (230 and 270 nm) is specifically absorbed. Light wavelengths, that are detectable for the human eye, were aborbed completely depending of ink concentration which explains the black, grey or whitish colour of the envelope. The function of the ink pigmentation of the envelopes remains obscure.

From our results it can be concluded that light is an important factor controlling hatching in *S.officinalis* under experimental conditions. Because LD transitions are also detected by black *Sepia* eggs, the embryos must be very sensitive to light and able to detect very low light intensities. Probably also in natural conditions light-dark transitions will synchronize hatching of *S.officinalis* because cuttlefish eggs are mostly found in relatively shallow and clear water (Von Boletzky, 1983; Chapters 8 and 10) where the daylight is only slightly attenuated (Poole and Atkins, 1937).

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INFLUENCE OF SALINITY ON EMBRYONIC DEVELOPMENT AND THE DISTRIBUTION OF *SEPIA OFFICINALIS* IN THE DELTA AREA*

ABSTRACT. The effect of salinity on embryonic development of *Sepia officinalis* (cuttlefish) in the Delta Area (South Western part of The Netherlands) was investigated in 1988/1989, and compared with data concerning the distribution of *S.officinalis* in the three main parts of this area called Oosterschelde, Westerschelde and Grevelingen. Embryos hatched in water collected at Yerseke (Oosterschelde), Vlissingen (Western part of the Westerschelde) and Bommenede (Grevelingen), *i.e.* at salinity values above 28.1 ‰, but not in water sampled at Hoedekenskerke and Hansweert (Middle and Eastern part of the Westerschelde where the salinities are below 22.0 ‰). Under laboratory conditions, using diluted Oosterschelde water, the highest hatching percentages of *S.officinalis* were found at salinities above 29.8 ‰. Some embryos hatched at a salinity value of 26.5 ‰ but hatching never occurred at salinities below 23.9 ‰. In embryos exposed to salinity changes during late embryonic development, the developmental rate decreased at 28.7 ‰ salinity or less. Below 22.4 ‰ malformations occurred. It can be concluded that salinity is an important factor limiting the distribution of *S.officinalis* in most parts of the Delta Area with the exception of the Western part of the Westerschelde and the Grevelingen.

INTRODUCTION

The common cuttlefish *Sepia officinalis* occurs in coastal waters and on the continental shelf at depths not greater than 150 m (Von Boletzky, 1983). *S.officinalis* generally moves inshore for spawning (Roper et al., 1984). After spawning the adults die, and in the late summer the juveniles move towards deeper waters. The Delta Area (South Western part of The Netherlands)(Fig. 10.1) is probably an important breeding territory for *S.officinalis*. Before 1953 the estuaries of this area were in open connection with the North Sea. In February 1953 large areas became inundated. Subsequently, the so-called Delta Plan was developed to protect the land against future aggression by the sea, *i.e.* the main estuaries were closed by high sea-walls. By 1987 the Delta Plan attained its achievement but the Westerschelde remained in open connection with the North Sea. This estuary is a very dynamic area with tidal differences of 4 to 5 m and a declining salinity gradient from the North Sea to the river Schelde (Nienhuis, 1982).

Before 1964 the Grevelingen was in open connection with the North Sea and with the rivers Rijn and Maas. The tidal difference amounted to 2.5 m and chlorinity of the water fluctuated between 10 and 17 ‰ (Peelen, 1967). For hydrotechnical reasons the Grevelingen estuary had to

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be closed by an upstream and a downstream dam. In 1964 the connection with the rivers was cut off by the inland Grevelingendam and a semi-enclosed estuary was left. In 1971 the mouth of the estuary was also closed (Brouwersdam), excluding the influence of the North Sea, and Lake Grevelingen originated. In 1978 the connection with the sea was reopened by perforating the downstream dam with an underwater sluice (capacity 100 to 140 $m^3 s^{-1}$). In 1983 another sluice came into operation in the upstream dam (capacity 100 $m^3 s^{-1}$), allowing an artificial flowing regime for the entire Lake Grevelingen (Bannink and Van Der Meulen, 1984).

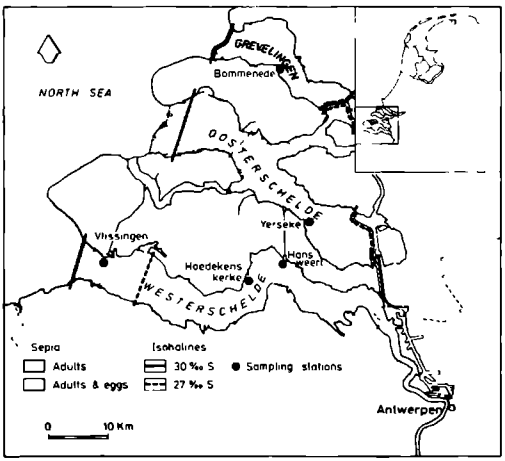

Fig. 10.1: The Delta of the rivers Rijn, Maas and Schelde. The five sampling places in experiment 1 and the areas where *Sepia officinalis* adults and/or eggs were found by fishermen are shown. Cf. Peelen, 1967.

During the last part of the Delta Plan a storm surge barrier was built. This barrier is a concrete construction with big rectangular holes which can be closed by enormous steel sliding doors. Because of the storm surge barrier the Oosterschelde remains in open connection with the North Sea so that marine animals can still reach this very important estuary. The Oosterschelde water is hardly mixed with freshwater and salinity levels are about the same as in the North Sea (Nienhuis, 1982).

Between the water of the Oosterschelde, Westerschelde and Grevelingen high salinity differences are found (Peelen, 1967; Van Boven, 1981, 1986; Merks et al., 1986a, b). These salinity differences probably affect embryonic development and subsequently the distribution of *S.officinalis* in the Delta Area, but this has hardly been investigated thus far. The aim of the present study was to investigate the effect of salinity on embryonic development and distribution of *S.officinalis* in the Oosterschelde, Westerschelde and Grevelingen.

MATERIALS AND METHODS

The study was carried out at the Delta Institute for Hydrobiological Research in Yerseke (The Netherlands). During spring and summer 1988 and 1989, eggs of *Sepia officinalis* from the Oosterschelde were collected by fishermen or from mature female adults kept in outdoor aquaria (capacity 1500 l), which were provided with running sea water coming directly from the Oosterschelde.

The experiments were performed in perspex aquaria (Fig. 10.2) placed in automatically controlled climate chambers kept constantly at a temperature of 17°C. The embryos developed

in a 12:12 h LD cycle The stage series of Lemaire (1970) was adopted. Three different experiments were carried out

Experiment 1 was carried out twice from June till August 1988 to investigate hatching in water sampled at several sites in the Delta Area. Four different spawns were used Eggs were removed from the 1500 l aquaria 48 hrs after oviposition and individual eggs of a spawn separated. Four eggs were placed at random in each tube so that every group started with 48 embryos. The perspex aquaria were filled with water sampled near the following places in the Delta Area: Hansweert, Hoedekenskerke and Vlissingen (Westerschelde), Yerseke (Oosterschelde) and Bommenede (Grevelingen) (Fig 10.1) The water was renewed at seven day intervals and sampled at high tide. Chlorinity, of both used and fresh water, was measured on the first day and subsequently at seven day intervals without further treatment, using a TTA 81 Autopipetting Titration Station (Radiometer, Copenhagen). Salinities were calculated using the hydrographical tables of Knudsen (1901)

The eggs were not disturbed, and after hatching daily counts were made of the number of juveniles. They were taken out of the aquaria for external morphological examination Empty egg shells were discarded to prevent growth of bacteria and fungi, and eggs which did not swell during the experiment were examined Both juveniles and embryos which had failed to hatch at the end of the experiment were fixed in Hollande's fixative (Gurr, 1962) and finally the hatching percentage in each group was calculated.

Experiment 2 was carried out in triplicate during summer 1989 to investigate embryonic development and hatching in diluted Oosterschelde water. As in experiment 1, embryos in very early embryonic stages were used Eggs from in total seven spawns were placed in the aquaria. For the controls (Group 1) 12 l of filtered Oosterschelde water was used. In all other groups (2 to 6) Oosterschelde water was diluted with distilled water to obtain a graded series between 10 and 31 ‰ S. The water was renewed at seven day intervals and salinity was measured as mentioned above. After hatching, juveniles were checked for malformations and condition, counted and finally fixed in Hollande's fixative. The remaining eggs were opened and examined at the end of the experiment or as soon as fungi and/or bacteria appeared on the egg surfaces.

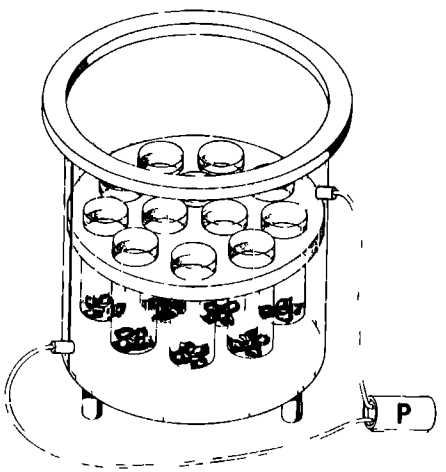

Fig. 10.2: One of the perspex aquaria in which the experiments were performed. The aquaria contained 12 l water (capacity 20 l). To facilitate the study of development and hatching of *Sepia officinalis*, a floating system was applied consisting of a tempex floater and 12 transparent, perforated plastic tubes (diameter 4 5 cm) Each aquarium was connected to a separate pumping device which enabled a closed circulation system without the need for extra aeration (P = pump).

In experiment 3 the effect of salinity on late embryonic development was investigated. In total three different spawns were used. They were left in the 1500 l aquaria until the majority of the embryos had reached stage 21 to 24. The eggs from the different spawns were separated and mingled After aquaria had been filled with water as in experiment 2, 80 eggs were placed in the tubes of each aquarium (Groups B to E). Group A was the control The experiment started on July 15, 1989, and immediately 20 eggs from each group were taken out and staged On July 22, 23 and 28, again 20 eggs were removed from each aquarium and the embryos were fixed after staging and checking their condition. Nearly all embryos were photographed.

To study the distribution of *S officinalis* in the Delta Area in total nine fishermen have been participating in our experiments. They were selected on basis of their particular working area which invariably covered parts of the Westerschelde, Oosterschelde or the Grevelingen In all parts of the area about the same fishing capacity was represented. During the spawning season of 1988 and 1989 the numbers of *S.officinalis* adults caught were registered, their condition checked and all spawns attached to nets or fish traps counted.

RESULTS

The results of experiment 1 are given in Fig. 10.3. In this experiment the highest hatching percentage (80.8 % ± 16.2) was found in water sampled at Yerseke, located near the Oosterschelde (Fig. 10.1). Also in water collected at Bommenede (Grevelingen) and Vlissingen (Western part of the Westerschelde)(Fig. 10.1) *S.officinalis* developed normally and high hatching percentages were found. In both areas salinity values were also rather high: between 28.1 and 30 1 ‰, as measured in the course of the experiment (Fig 10.3A). All juveniles had reached stage 30 (Lemaire) before hatching.

During 1988 and 1989 adult specimens of *S.officinalis* were found everywhere in the Oosterschelde (Fig 10.1). In both years most adults were caught during May, 252 in total. In contrast during June and July, 185 and 25 adults were found, respectively. In all subtidal parts of the Oosterschelde fishermen found spawns attached to their nets during May, June and July of both years. Apparently, *S.officinalis* has no preferential spawning area in the Oosterschelde.

In the Westerschelde *S officinalis* adults were only caught in close vicinity of the North Sea (Fig. 10.1), in total four during May and three during July. This is considerably less than in the Oosterschelde. *S.officinalis* spawns or adults were not found in other parts of the Westerschelde. The numbers of adults found in the Grevelingen during 1988 and 1989 were even smaller than in the Westerschelde. Only one adult was caught during April 1988 and two during May of the same year. Adults were not observed in 1989. No spawns have been recovered in 1988 or 1989.

In experiment 1 hatching never occurred in sea water sampled at Hoedekenskerke or

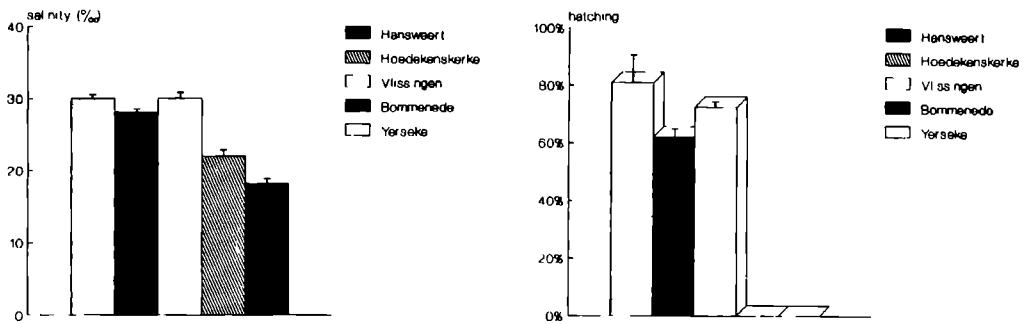

Fig. 10.3: Hatching of *Sepia officinalis* in water from various sites in the Delta Area. A. average salinity values of the sea water from the various sampling sites of experiment 1 B hatching percentages.

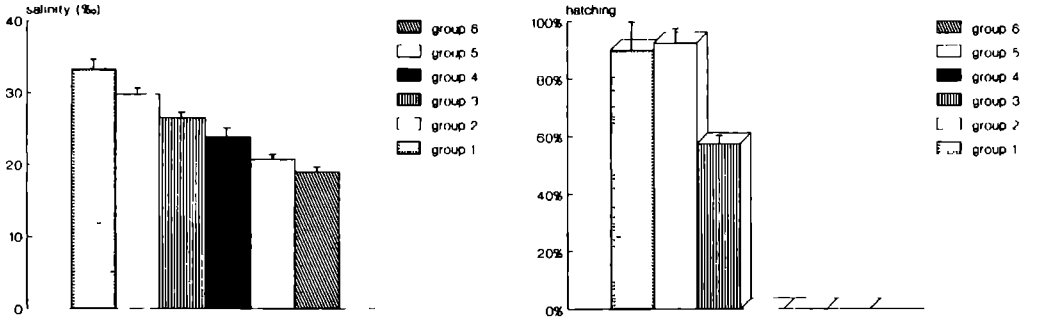

Fig. 10.4: Hatching of *Sepia officinalis* in diluted Oosterschelde water. A: average salinity values of the water in Groups 1 to 6 during experiment 2. B: hatching percentages.

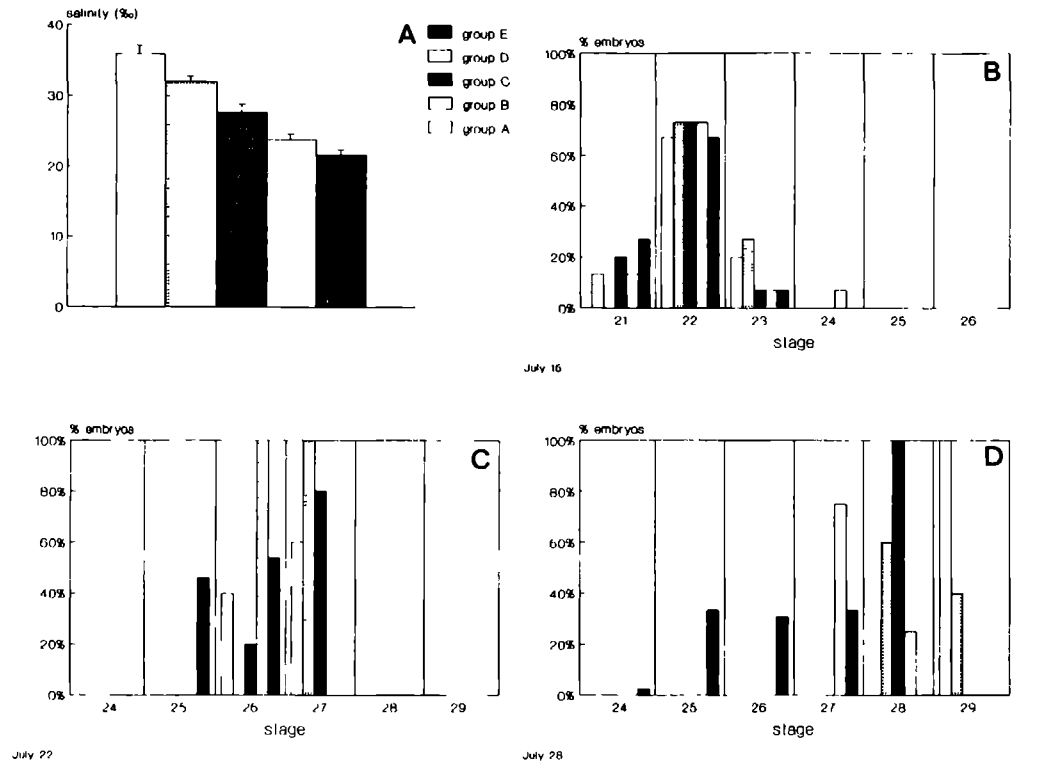

Fig. 10.5: The effect of salinity on late embryonic development of *Sepia officinalis*. A: average salinity values of the water during experiment 1 in Groups A to E. B: developmental stage of the embryos in the various groups when experiment 3 started (July 15, 1989). C: distribution of the embryonic stages after 8 days. D: development after 14 days.

Hansweert (Fig. 10.3B). This became already apparent 20 days after the start of experiment 1. In normally developing eggs the envelope is dilated by the chorion which expands because of the increasing osmotic pressure of the perivitelline fluid (PVF)(Lemaire, 1971). In water sampled at Hoedekenskerke and Hansweert the eggs retained about the same size as at the beginning of the experiment, *i.e.* 48 hrs after oviposition. After 30 days the eggs were opened and their contents examined. All embryos had died in very early developmental stages (stage 3 to 15, Lemaire) and had opaque, whitish yolk sacs while normally the yolk is almost transparent. In some cases the PVF was filled with a white, flaky substance and the embryo could not be recognized. As shown in Fig. 10.1, Hansweert is located in the more eastern part of the Westerschelde, close to Antwerpen and the river Schelde. Salinity values in this area are low, on average 18.3 ‰ S during the experiment (Fig. 10.3A). Also in Hoedekenskerke low salinity values (22.0 ‰) were measured. During 1988 and 1989 *S.officinalis* adults or spawns were never found near Hansweert or Hoedekenskerke (Fig. 10.1).

In experiment 2, a salinity gradient was imitated using diluted Oosterschelde water. The results of this experiment are given in Fig. 10.4. In Groups 1 and 2 high hatching percentages were found (Fig. 10.4B). All juveniles had reached stage 30 (Lemaire) before hatching. Salinity values of the water in Groups 1 and 2 were 33.3 ± 1.8 and 29.8 ± 2.0 ‰ S respectively (Fig. 10.4A). In Group 3, 57.5 % of the embryos hatched. Of the remaining embryos, 30 % had died in stage 30 and 7.5 % were still alive but in poor condition at the end of the experiment; the embryos did not move

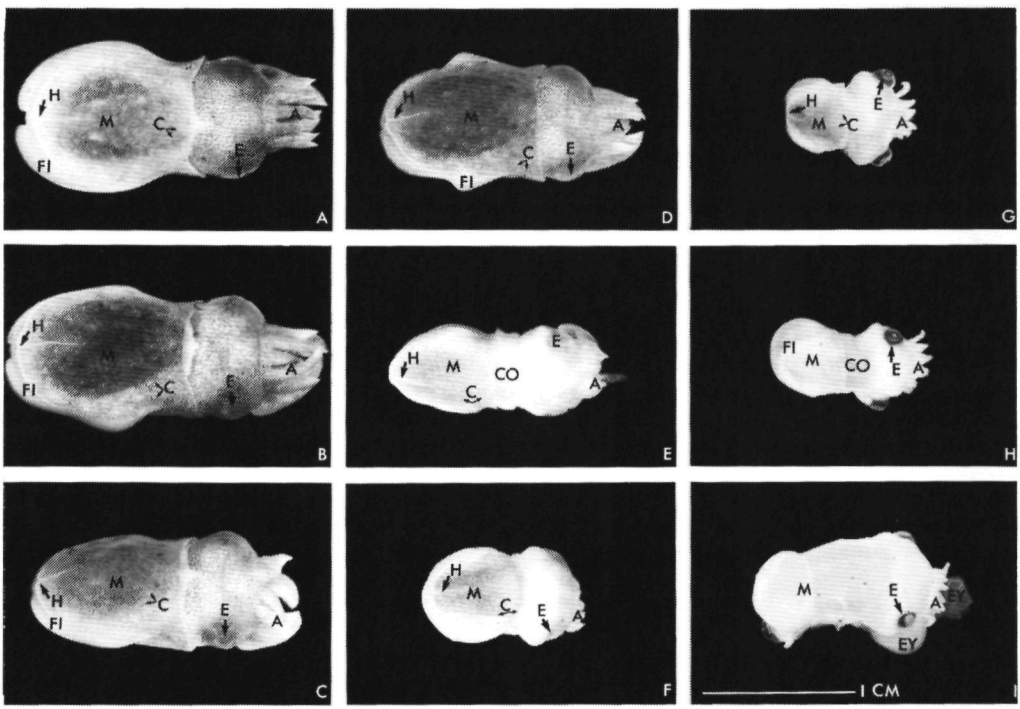

Fig. 10.6: Impact of salinity on development and morphology of *Sepia officinalis* embryos from Groups A to E in experiment 3 on July 28, 1989, *i.e.* 14 days after experiment 3 started. (A) Group A. (B) Group B. (C) Group C. (D and E) Group D and (F to I) Group E. For salinity see Fig. 10.5A. For the distribution of developmental stages on July 28, see Fig. 10.5D. Abbreviations are: A=arms, C=chromatophore, CO=collar, E=eye, EY=external yolk sac, FI=Fin, H=Hoyle's organ, M=mantle.

upon being touched and were whitish instead of brown. In Group 3 the salinity value of the water was 26 5 ± 1.3 ‰ S. No hatching occurred in Groups 4, 5 and 6 (Fig. 10.4B). After 14 days the eggs of these groups were removed from the aquaria. It appeared that all of them had died in very early embryonic stages. The salinity values in these groups varied between 23.9 ± 1.4 and 19.0 ± 0.6 ‰ S during the experiment (Fig. 10.4A).

The results of experiment 3 are represented in Figs. 10.5 and 10.6. The average salinity values of the water in each group are given in Fig. 10 5A As experiment 3 started on July 15, most embryos had developed to stage 22 (Fig. 10 5B). After 7 days on July 22, it became clear that the developmental rate in each experimental group differed. In Groups A, B and C most embryos had reached stage 27 while in Group D all embryos sampled had developed to stage 26. In Group E about half of the embryos had reached stage 26; the remaining embryos had developed to stage 25 (Fig. 10 5C). On July 28, the developmental rate differences became even more striking. In Group A all embryos had reached stage 29; in Group B only 40% of the embryos had reached stage 29 and 60% developed to stage 28; all embryos in Group C had reached stage 28; while in Group D most embryos had only developed to stage 27. The lowest developmental rate was found in Group E. Here only one third of the group had reached stage 27, while all other embryos had got only as far as stage 24, 25 or 26 (Fig. 10.5D).

In Fig. 10.6, embryos from each group of experiment 3 placed in fixative on July 28, are represented. Embryos from Groups A (Fig. 10.6A) and B (Fig. 10.6B) had reached stage 29 (almost 30) and showed no externally visible malformations. Also, stage 28 embryos from Groups C (Fig. 10.6C) and D (Fig. 10.6D) had a normal appearance In Fig. 10.6E a stage 27 embryo from Group D is shown Evidently, the mantle has not reached the normal length. As a result the collar becomes visible. Figs. 10.6F to I show embryos from Group E. The embryos had developed slowly and about half of them presented externally visible malformations such as short mantles (Fig. 10.6H), while others were profoundly deformed (Fig. 10.6I).

On July 28 also the diameter of the eggs from the various groups differed. In Group A the diameter varied between 1.0 and 1.1 cm and from each egg 2.3 ± 0.4 ml PVF could be obtained. In Group E the egg diameter varied between 6.9 and 7.6 cm and only 0.8 ± 0.3 ml PVF could be sampled from each egg. The PVF from eggs in Group A was transparent and all eggs were buoyant. In Group E eggs were instead soft and very often white flaky substances (probably vitellus) were found in the PVF.

DISCUSSION

From experiment 2 it can be concluded that the highest hatching percentages of *S officinalis* are found at salinities above 29 8 ‰. Some embryos could hatch at a salinity value of 26.5 ‰ but no hatching occurred at salinities below 23.9 ‰. These results are in agreement with experiment 1; embryos hatched in sea water sampled at Yerseke, Vlissingen and Bommenede, *i.e.* at salinity values above 28.1 ‰ In water collected at Hoedekenskerke and Hansweert (salinities below 22 03 ‰) hatching was never observed. From the results of experiments 1 and 2 it can be concluded that the hatching percentages in water from the various sampling sites were directly related to the salinity values of the water.

According to Palmegiano and D'Apote (1983) hatching of *S.officinalis* from the Gulf of Lesina (Italy), is completely inhibited below 25 ‰ S at temperatures of 15°, 18° and 21°C. This is in agreement with our studies (carried out at 17°C) in which embryos still managed to hatch at salinity values of 26.52 ‰ (compare with experiment 2), whereas no hatching occurred at lower salinities. In 1966 Choe working with species from the Sea of Japan found that the eggs of *Sepia esculenta* yield a 58 to 66 % hatch and *Sepiella maindroni* a 50 to 63 % hatch at salinities of 25.4 ‰ or higher. A hatch greater than 80% can be expected for *S.esculenta*, *Sepia subaculeata*, *S.maindroni*, *Sepioteuthis lessoniana* and *Euprymna berry* if the eggs are kept in running sea water exceeding 27.1 ‰ S. Also these observations resemble our results with *S.officinalis*. D'Aniello et al. (1986; 1989) found that *Loligo vulgaris* from the Bay of Naples will not develop at a salinity below 28 ‰ or higher than 47 ‰. Apparently, *S.officinalis* is slightly more tolerant to salinity variations.

In experiment 3 the developmental rate of embryos developing at salinity values of 28.7 ‰ or

less, slowed down. Osmotic stress probably demanded a lot of energy which could not be used for development. Below 22.4 ‰ S malformed embryos were found Salinity changes imposed during late embryonic development had no immediate lethal effect: after 14 days embryos developing in water with a salinity value of 19 6 ‰ were still alive, but none of them hatched. These results are in agreement with observations by Von Boletzky (1983), that young *S.officinalis* can survive for some time at salinities around 18 ‰ if slowly acclimatized. For normal embryonic development salinities above 25 ‰ are required.

From our results it can be concluded that salinity is an important factor regulating the distribution of *S.officinalis* in most parts of the Delta Area, except the Western part of the Westerschelde and the Grevelingen Our investigations indicate that high hatching percentages are obtained in water sampled near Vlissingen, but in this area eggs were not found and adults rarely appeared. This can probably be explained by the hydrodynamic character of this area The common cuttlefish usually attaches its eggs to marine plants, branches of gorgonians, twigs or similar objects (Bott, 1938). As Grimpe (1928) reported, it is exceptional that eggs are laid on non-sedentary animals such as crabs. In the Westerschelde all fixed objects are exposed to salinity fluctuations (Saeijs, 1977) Although *S officinalis* is known to be relatively tolerant to salinity variations [Mangold-Wirz (1963) has found some adults in the coastal lagoons in the Mediterranean at salinity values of 27 ‰], eggs attached to fixed supports in the Westerschelde are exposed to osmotic stress, leading to low hatching percentages (cf. experiments 1 and 3). Probably the adults caught in the Westerschelde could survive in this area because they could comply with the salinity fluctuations. Other factors like navigation and unsuitable spawning places (Saeijs, 1977; Nienhuis, 1982) could also limit the abundance of *S.officinalis* in the Westerschelde.

The water in the Grevelingen appears very suitable for the development of cuttlefish embryos, although likewise in this area adults rarely appeared and eggs were never found Before the downstream dam was built, *S.officinalis* eggs and adults were very common in the Grevelingen (Kaas and Ten Broek, 1942, B J. Steketee pers comm.). As the mouth of the Grevelingen estuary was closed in 1971, *S.officinalis* completely disappeared. In 1978 the downstream dam was reopened, but because of possible stratification danger, the underwater sluice is closed between April and October (H. Verheij, pers. comm.). In the Delta Area the first *S officinalis* adults appear in early spring and spawning occurs in shallow waters, mainly between April and July (Roper et al., 1984). It is evident that only the very early migrating adults of *S.officinalis* can reach the Grevelingen in time. Their congeners, which migrate later, will probably spawn in the Oosterschelde Our results indicate that in water from the Oosterschelde high hatching percentages are obtained and adults and spawns are very common. Evidently, the cuttlefish easily pass through the large holes in the storm surge barrier.

Finally, we can conclude that at this moment the Oosterschelde is the most important breeding territory of *S.officinalis* in the Delta Area In the Grevelingen *S.officinalis* will probably become more common when the underwater sluice doors in the downstream dam are kept open during spring and summer. Water conditions, as well as the high percentages of prey animals (Bakker, 1978; Nienhuis, 1978) and the extensive seagrass ecosystems (Nienhuis, 1978) in Lake Grevelingen make this area suitable as a breeding area for *S.officinalis*.

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THE EFFECT OF ABRUPT SALINITY CHANGES ON LATE EMBRYONIC DEVELOPMENT AND HATCHING OF *LOLIGO VULGARIS* IN THE DELTA AREA*

ABSTRACT. Starting at stages X-XII (Naef) the effect of abrupt salinity changes on embryonic development and hatching of *Loligo vulgaris* (European squid) embryos from the Oosterschelde (S ± 31 ‰) was investigated. During the experiments no difference in salinity value was found between the perivitelline fluid (PVF) and the surrounding sea water. When the salinity of the water was abruptly reduced to 28.7 ‰, hatching and survival percentages were still high but below 28.7 ‰ the hatching percentages dropped. Only a few embryos developed normally after being exposed to salinities between 22 and 25 ‰. In embryos with deranged development abnormalities in mantle-length were most striking. Embryos kept at 19 ‰ salinity, after six days showed mantle deformations and many developed enormous bulbs on the head. Invariably deformed embryos showed internal malformations, except in the eye and the statocyst. The internal yolk sac was grossly enlarged. No hatching took place at 19 ‰ salinity. On the whole our results permit to conclude that salinity probably affects the distribution of *L.vulgaris* in the Delta Area.

INTRODUCTION

According to investigations of Paulij et al. (Chapter 10 of this thesis) in 1988 and 1989, spawns of *Sepia officinalis* (common cuttlefish) were regularly found in the Oosterschelde, an inlet of the North Sea and part of the Delta Area (South Western part of the Netherlands). In other parts of this area, like the Westerschelde estuary, where North Sea water is mixed with freshwater from the river Schelde (Rijstenbil, 1987), and Lake Grevelingen, cuttlefish spawns were never observed. From these observations and from experimental evidence it was concluded that salinity was the most important factor limiting the distribution of *S.officinalis* in most parts of the Delta Area with the exception of the Western part of the Westerschelde and the Grevelingen.

The same fishermen who had contributed to the previous study (see Chapter 10 of this thesis) also presented evidence that *Loligo vulgaris* (European squid) spawns and adults only in the Oosterschelde. Adults were mostly found during May and June (378 and 1086 in 1988, respectively). These observations were very similar to the data collected for the cuttlefish. It seems that salinity differences also affect the distribution of *L.vulgaris* in the Delta Area, but the influence of salinity on embryos and juveniles of *L.vulgaris* from this area has not yet been investigated.

Because the sea water salinity conditions of the Grevelingen are very similar to those of the Oosterschelde, we tried in our experiments to reproduce the natural salinity of the Westerschelde. The Westerschelde estuary is a very dynamic area with tidal differences of 4 to 5 m and a declining salinity gradient from the North Sea to the river Schelde (Nienhuis, 1982).

* Chapter 11 is the modified version of Paulij, W.P., Hummel, H., Denucé, J.M (submitted) The effect of abrupt salinity changes on late embryonic development and hatching of *Loligo vulgaris* in the Delta Area (South Western part of The Netherlands).

In the present study *L vulgaris* embryos were exposed to experimentally produced abrupt differences in salinity *L.vulgaris* females usually attach their eggs to debris, fishing nets or on various other hard objects on sandy to muddy bottoms (Mangold-Wirz, 1963; Roper et al., 1984) In the Westerschelde all fixed objects are exposed to considerable tidal and/or seasonal salinity fluctuations (Saeijs, 1977). The aim of the present study was to investigate the relative tolerance of developing *L.vulgaris* embryos to these salinity "shocks".

MATERIALS AND METHODS

The study was carried out at the Delta Institute for Hydrobiological Research at Yerseke (The Netherlands) with embryos of *Loligo vulgaris*. We completely depended on spawns collected by fishermen during May and June 1988 and 1989. Because the embryos had reached various stages of embryonic development at the time of collection, we started our investigations about halfway embryonic development (*i.e.* as soon as all embryos within one egg string has reached stage X-XII (Naef, 1923; 1928) in order to synchronize the experiments. Stages X-XII are rather easy to identify without removing the embryo from its egg capsule Variation in the embryonic stages within one egg capsule can be explained by the position of the egg in a strand: distal eggs develop faster than proximal ones (Chapter 8). To avoid variation in developmental age, separating the eggs can be an option, but using separated eggs is not very similar to the natural situation because part of the surrounding jelly layer must be removed. In normally developing eggs the perivitelline fluid (PVF) greatly increases in volume during embryonic development, due to water uptake from the surrounding jelly (Von Boletzky, 1987b) As this process is mainly an osmotic event (Lemaire, 1971) salinity differences in the environment play obviously an important role. Until the embryos had reached stage X-XII (Naef) the spawns were suspended vertically in an aquarium (capacity 1500 l) provided with running sea water coming directly from the Oosterschelde.

The experiments were performed in 5 perspex aquaria filled with 10 litres of filtered water (capacity 20 l) Each aquarium was connected with a separate pumping device to obtain a closed circulation system without the need for additional aeration. The aquaria were placed in an automatically controlled climate chamber which was kept constantly at 15°C.

Dilutions were made by mixing filtered Oosterschelde water (S about 31 ‰) with distilled water. In this way a graded salinity series was obtained (Groups 2 to 5). Control aquaria for Group 1 were filled with 10 litres of filtered Oosterschelde water. The water was renewed every 4 days. Chlorinity was measured at the onset of each experiment and subsequently every fourth day, without further treatment, using a TTA 81 Autopipetting Titration Station (Radiometer, Copenhagen). Salinities were calculated using the hydrographical tables of Knudsen (1901).

After the majority of the embryos had reached stage X-XII (Naef) the egg strings were suspended vertically in the perspex aquaria. In 1988 a total of five different spawns were used One string of each spawn was placed in an aquarium (*i e.* more than 450 embryos per experimental group). The embryos were left undisturbed until they had reached stage XX (*i e* hatching stage). The strings were then transferred to cuvettes (30x30x10 cm) containing the same sea water as the aquaria, in order to count the number of juveniles. After all embryos had hatched (or died) the hatching percentage was calculated. ([Hatching percentage = number juveniles · initial number of embryos] x 100).

The experiment was repeated in 1989. This time, four different spawns were used. Again each aquarium received one string of every spawn. The water was changed every other day for 12 days. The first day and thereafter every second day fifty eggs were removed (N=350 group ^{1}) The perivitelline fluid of 50 eggs from each Group was collected on days 6, 8 and 10 (N=150 Group ^{1}). To collect the PVF the eggs were separated from the strings with dissection needles, placed in a dry petri dish on melting ice and the chorion was torn up with two watchmaker tweezers. The PVF was collected using a Gilson pipette. Chlorinity values of the PVF and the water were measured as indicated above. After being examined for possible morphological defects the embryos were fixed in Hollande's fixative (Gurr, 1962) and photographed. The embryos were then embedded in paraffin and 5 µm thick longitudinal sections were stained with haemalum-eosin (HE) for microscopic examination.

In each aquarium one egg string was left undisturbed until the embryos reached hatching stage XX. These strings were then transferred to cuvettes (like the strings in 1988) and the external morphology of every juvenile was checked before and after fixation. The hatching percentage was then calculated as indicated above.

RESULTS

The average salinities of the water and the PVF in each experimental group are given in Table 11.I. Note that in Group 5 the salinity of the PVF was measured only once, on day 6, because the embryos died very quickly during the experiment. The egg capsules in this group did not expand during development, in contrast to eggs developing in filtered full-strength Oosterschelde water. Instead they remained small and soft. Consequently the total amount of PVF within one egg was smaller than in the controls.

The hatching percentages within each experimental group are given in Fig. 11.1. The highest percentages were found in sea water with salinities ranging between 28.7 and 31.6 ‰. In each experiment hatching percentages dropped abruptly at salinities below 28.7 ‰.

The juveniles in Groups 1 and 2 showed chromatophore expansions and inking if they were removed from their egg capsules. They all lived longer than 24 hrs. In contrast, the individuals in Group 3 all died within 24 hrs. The juveniles in Group 4 died already a few hours after hatching.

During normal embryonic development embryos of *L.vulgaris* are nearly transparent, and after

Table 11.I. Salinity values of the sea water in 1988 and 1989 and of the peri-vitelline fluid (PVF).

Group	Salinity value sea water 1988	Salinity value sea water 1989	PVF 1989
1	31.6 ± 0.9	30.1 ± 0.7	31.0 ± 1.4
2	28.7 ± 0.7	28.8 ± 0.7	27.6 ± 4.6
3	25.2 ± 0.2	25.1 ± 0.8	25.8 ± 1.1
4	22.1 ± 0.1	22.1 ± 0.7	24.2 ± 5.6
5	19.3 ± 0.2	19.2 ± 0.1	23.5*

* measured only once (see Results)

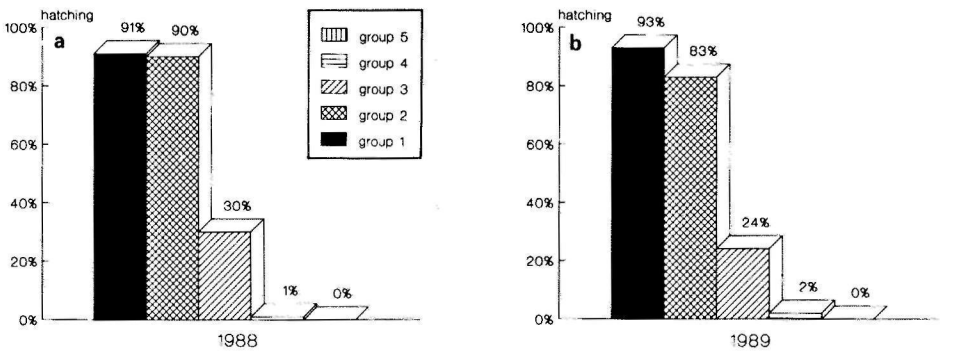

Fig. 11.1. Hatching percentages of *Loligo vulgaris* in the experimental groups in 1988 (a) and 1989 (b). For salinity values of the water see Table 11.I.

stage XII-XIII body movements and contractions of the mantle can easily be observed. Stage XVII-XX embryos normally show chromatophore expansions when disturbed. In our experiments the head and in some instances the entire body of dying embryos appeared opaque and the embryos did not react after being touched. In this paper these mostly moribund embryos are referred to as embryos in a "bad condition". The opaqueness of the embryos in Figs. 11.3 and 11.4 is caused by fixation.

In Fig. 11.2 the impact of different salinity conditions on the embryonic development of *L.vulgaris* in 1989 is given. To describe the events we refer to day numbers instead of developmental stages because in abnormally developed embryos the morphological features on which the

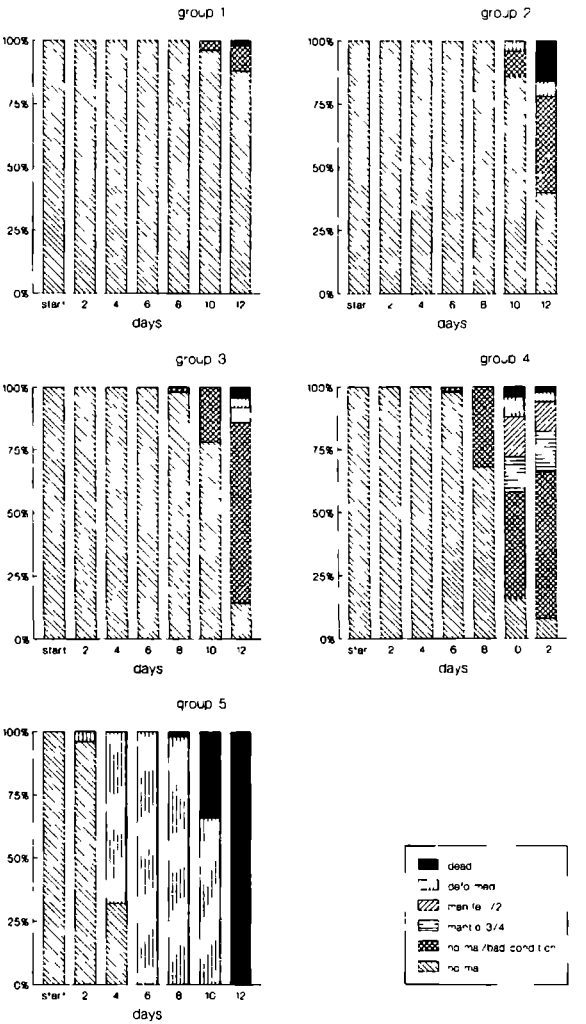

Fig. 11.2. Embryonic development of *Loligo vulgaris* from stage X-XII (Naef) on in water with different salinities. For salinity values of the sea water in each experimental group see Table 11.I (1989). Each bar represents 50 embryos.

staging system is based have not come to expression or cannot be found at the time or in the way they are expected to appear.

In Group 1 the majority of the embryos developed normally (Fig. 11.3A). Only two out of fifty embryos appeared in a bad condition ten days after the experiment started. Their morphology was not impaired but they were opaque and whitish, and remained motionless after the chorion and the envelope had been removed. On day 12 one embryo had died. In Group 1 hatching started already 9 days after the begining of the experiment while the majority hatched after 11 days.

In Groups 2 and 3 hatching started after day 10 and 9 respectively. In both groups the vitality of the embryos declined during the experiment. In Group 3, the first opaque embryos were found

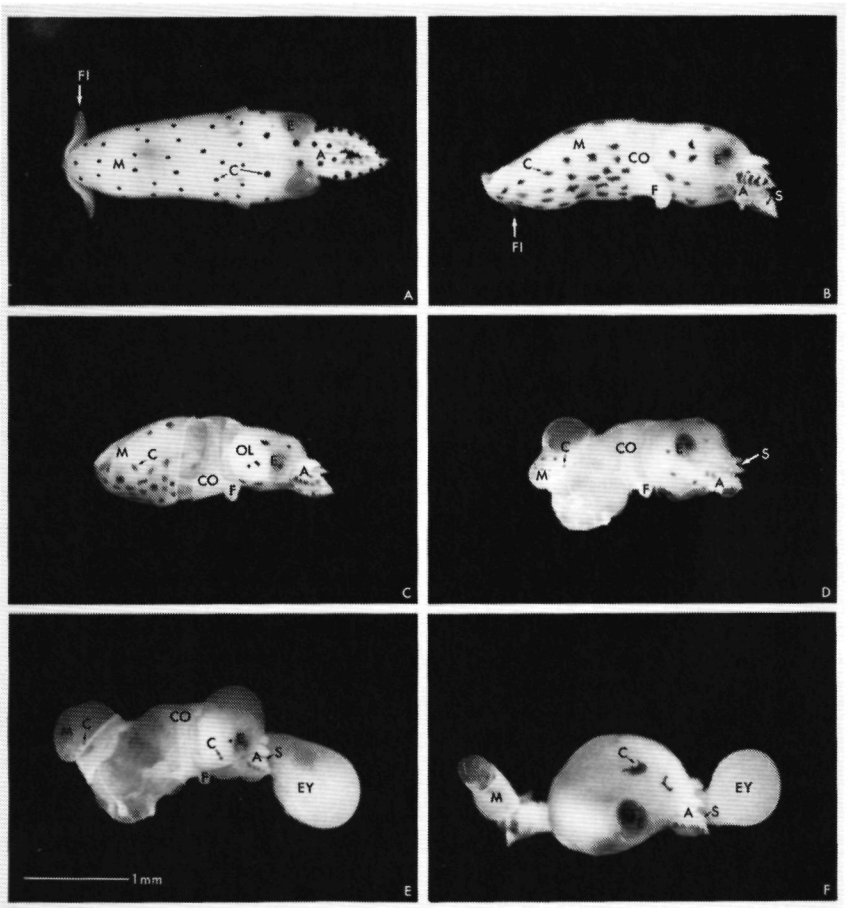

Fig. 11.3. Preserved *Loligo vulgaris* embryos from the different experimental groups. 3A: normally developed juvenile immediately after hatching. 3B: embryo from Group 3 with 3/4 of the usual mantle-length. Part of the collar is visible. 3C: embryo with only 1/2 of the normal mantle size. 3D: embryo from Group 5 with a deformed mantle. 4E: embryo from Group 5 with a deformed mantle and a bulge on the head. 3F: completely malformed embryo with a strongly reduced body from Group 5. Abbreviations: A=arms, C=chromatophore, CO=collar, E=eye, EY=external yolksac, F=funnel, FI=fin, M=mantle, S=sucker.

on day 8. At day 12 three embryos had developed normally, in spite of the fact that the mantle-length had reached only about 3/4 of the normal size (Fig. 11.3B). The collar and half of the funnel were visible. None of these embryos hatched, although Hoyle's organ was distinguishable. On day 12 most of the embryos were moribund.

In group 4 only four out of fifty embryos had developed normally on the last day of the experiment. The first moribund embryo was found on day 6, increasing up to 16 on day 7 and 29 on day 12. On day 10 the first embryos with 3/4 of the normal mantle-length were seen (Fig. 11.3B). Embryos with a mantle-length of 1/2 the normal size (Fig. 11.3C) were found on day 10 and 12.

In Group 5 the first deformed embryos were already observed on the second day of the experiment. On day 6 all embryos showed conspicuous malformations. Whereas only 50% had only deformed mantles (Fig. 11.3D), most embryos had also developed enormous bladders on the head (Fig. 11.3E). Some embryos were completely malformed, showing heads of abnormal size and shape and strongly reduced bodies (Fig. 11.3F).

In Fig. 11.4 the dorsal side of normally developed embryos at stage XIII and XVII and of malformed embryos from Group 5 are shown. The external bilateral symmetry of the mantle is preserved, despite the malformations. In all embryos shown, Hoyle's organ was visible and fins had been formed.

Para-sagittal and medio-sagittal sections of a normally developed embryo are shown in Figs. 11.5A and B. Sections of an embryo with a mantle half the normal size are represented in Figs. 11.5E and F (cf. Fig. 11.3C). In these embryos the anterior part of the mantle is lacking because the mantle is present in an unexpanded state. The chromatophore pattern is compressed. Only

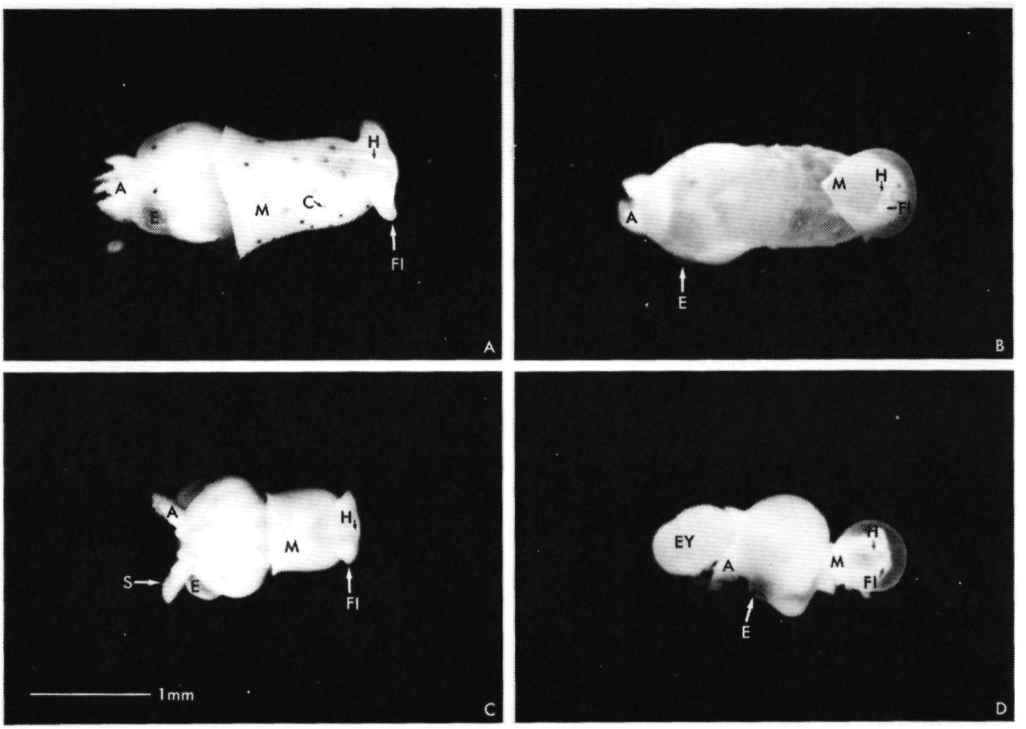

Fig. 11.4. Dorsal side of preserved normal *Loligo vulgaris* embryos in stage XVII (Fig. 11.4A) and at stage XIII (Fig. 11.4C) and malformed embryos of Group 5 (Figs. 11.4B and D). The external bilateral symmetry of the mantle is preserved, despite the deformations. For abbreviations see Fig. 11.3.

a few nerves of the stellate ganglion had developed completely. They were situated more posteriorly in the body than in normal embryos. Parts of the brain and the digestive system could be discerned despite incomplete development. In embryos with a shortened mantle, epidermal differentiations like Hoyle's organ, the ciliated cells and the mucous cells appeared like in control

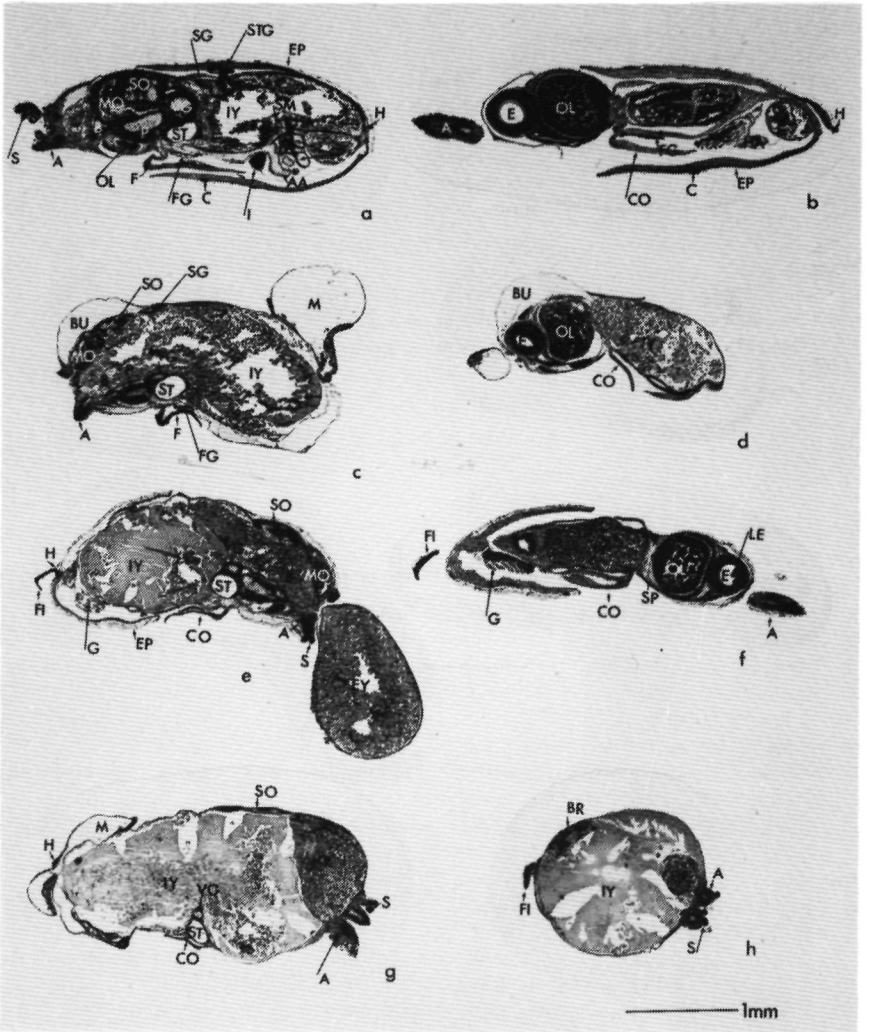

Fig. 11.5. Medio-sagittal (left) and para-sagittal (right) sections of a normally developed *Loligo vulgaris* embryo (Figs. 11.5A and B), an embryo with a deformed mantle and a bulge on the head (Figs. 11.5C and D), an embryo with half the normal mantle-length (Figs. 11.5E and F) and a completely malformed embryo (Figs. 11.5G and H). A = arms, AA = aorta anterior, B = brachial ganglion, BR = brain rudiments, BU = bulb, C = chromatophore, CO = collar, E = eye, EP = epidermis, EY = external yolk sac, F = funnel, FG = funnel gland, FI = fin, G = gills, H = Hoyle's gland, HA = heart, HR = head region, I = ink sac, IY = internal yolk, L = liver, LE = lens, M = mantle, MO = mouth, OL = optic lobe, P = pedal ganglion, S = sucker, SG = salivary gland, SM = stomach, SO = supraoesophageal lobes, SP = sinus cephalicus, ST = statocyst, STL = stellar ganglion, VG = visceral ganglion.

embryos The epidermal cells in the mantle edge showed an abnormally high number of nuclei compared to the total number of cells. This was also the case in embryos with mantles 3/4 of the normal length (cf. Fig. 11.4B).

Sections of an embryo with a deformed mantle and a bulge on the head are shown in Figs. 11.5C and D (cf. Fig. 11.3E). The bulge appeared edematous. The stellate ganglion remained rudimentary. The gills were always small and underdeveloped, protruding out from the posterior part of the body. Rudiments of the digestive and the vascular systems were also found. In the mantle epithelium Hoyle's organ and the ciliated cells appeared but most of the muscular tissue, which is normally located beneath the mantle epithelium, was lacking The eyes, the collar and the statocyst did not show anomalous development.

In malformed embryos (Figs. 11.5G and H)(cf. Fig. 11.3F) only a few structures such as the fins, Hoyle's organ and the ciliated cells, had developed in a normal way. Although the cells of Hoyle's organ were filled with granules, none of these embryos hatched. Most of the dermal muscle was absent, except where chromatophores appeared The funnel and the funnel gland were visible. The collar remained below the normal size. The visceral ganglion appeared underdeveloped, lying just above the statocyst In some malformed embryos rudiments of the pedal, brachial and buccal ganglia, and of the optic lobes could be discerned Most of the digestive organs seemed to be absent altogether. The circulatory system was affected In most embryos the arms remained shorter than in the controls but suckers were visible. Only the eyes and the statocysts had developed in the same way as in control embryos. As the eyes always develop laterally they do not appear in the para-sagittal section.

Finally, the HE sections showed that in all, more or less deformed embryos, the internal yolk sac was enlarged.

DISCUSSION

The present study indicates that high hatching and survival percentages are obtained when *L.vulgaris* embryos are exposed during late embryonic development to an abrupt salinity drop from about 31‰ to 28.7 ‰. At lower salinities there is a sharp decrease in hatchability and survival.

When the embryos were exposed to a single salinity drop to values below 25 ‰, many showed conspicuous malformations except in structures which had (partly) been formed before stage X-XII (Naef), such as the eyes (Arnold, 1971; Fioroni, 1978), the nervous system, the statocysts (Fioroni, 1978) and epidermal differentiations such as the fins (Von Boletzky, 1982b), Hoyle's organ (Yung Ko Ching, 1930; Chapters 2 and 3 of this thesis), and the ciliated cells (Arnold and Williams-Arnold, 1980; Chapters 2 and 3 of this thesis) Our results indicate that, like in embryos of *S.officinalis* (Chapter 10), reducing the salinity did affect further growth of these structures Most likely, the osmotic stress demanded a lot of energy which could not be used for development The most obvious aberration of abnormally developed embryos was the enlarged size of the internal yolk sac. Most abnormalities can be related to this initial defect *i.e* inability of the mantle to cover internal organs and defective development of internal (intestinal) structures. This is a typical feature of disturbed cephalopod embryonic development.

Our results can be compared with previous data of D'Aniello et al. (1986, 1989) concerning *L.vulgaris* embryos from the Mediteranean According to these investigations the embryos develop normally in natural sea water from the Bay of Naples, which has a salinity value of about 38 ‰. When 12-14 days before hatching the embryos were exposed to salinities of 28 ‰ or less, no hatching took place (D'Aniello et al., 1989) From these and our results it can be concluded that *L.vulgaris* embryos develop normally in a wide range of salinity values *i e.* between 38 and 30 ‰, when they are exposed to these salinities from the moment of spawning. Development and hatching are affected when *L.vulgaris* embryos are exposed to single salinity shocks of more than 6 ‰ S about halfway the embryonic development The relative tolerance of *L vulgaris* embryos to salinity shocks depends on the embryonic stage. It increases with proceeding embryonic development. According to D'Aniello et al. (1986; 1989) 40-60 % of the mediteranean embryos, developed until 2-3 days before hatching, hatched at a salinity value of 28 ‰.

From our results it can be concluded that embryos of *L.vulgaris* will neither develop nor hatch

normally in the Westerschelde estuary because in most parts of the Westerschelde salinity fluctuations between 5 and 10 ‰ are not exceptional (Peelen, 1967). These salinity fluctuations can affect the embryo because the properties of the PVF, as far as salinity is concerned, remain equal to the surrounding sea water (cf Choe, 1966, D'Aniello et al., 1986). Near the North Sea, where salinity fluctuations are less high, more suitable water conditions for embryonic development appear but probably other factors like navigation, pollution and unsuitable spawning places will limit the abundance of *L.vulgaris* adults in this area (Saeijs, 1977; Nienhuis, 1982).

Although our results indicate that *L.vulgaris* embryos probably develop normally in the Grevelingen (S about 28 ‰), adults and spawns never appeared in this area in 1988 and 1989. The same observations were made with *S.officinalis* and it was concluded that the absence of this species was due to the early closure of the downstream dam in spring (Chapter 10). This conclusion probably also holds for *L.vulgaris*. The European squid approaches the French coast in spring and migrates from May through June further north into the North Sea when the underwater sluice doors of the downstream dam are already closed till fall (Roper et al., 1984).

In the Oosterschelde *L.vulgaris* and *S.officinalis* spawns and adults are found because the animals can reach this area via the large holes in the storm surge barrier (Chapter 10). The absence of salinity fluctuations together with suitable water conditions contribute to the fact that at this moment the Oosterschelde is the most important breeding territory of the common cuttlefish and the European squid in the Delta Area.

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We are grateful to Mr. R.H. Bogaards of the Delta Insitute for Hydrobiological Research in Yerseke (The Netherlands) for contacting the fishermen and to Messrs. E.J. van Hannen, A.J.M. Coenen, C.M. de Rooy and J.J. Sinke for practical help. One of us (W P P) is particularly indebted to Prof J.Z. Young (Oxford University) for explaining the morphology of the cephalopod embryo. Thanks are also due to Messrs. G.H.M. Dekkers, M.C.J. Groos, R.H.G. Kleingeld and H.J.M. Spruyt for preparing the illustrations and the photographic part of this work, to all fishermen who cooperated in this project, to Mrs F. van Lent for advice and to Mrs. E.A.J. Derksen for secretarial assistance.

The first part of this thesis describes structural and biochemical studies on ectodermal differentiations and the hatching enzyme of embryos and early juveniles of the cephalopods *Loligo vulgaris* (European squid), *Loligo forbesi* (veined squid) and *Sepia officinalis* (common cuttlefish). In the second part three environmental factors (copper, light and salinity) probably controlling or affecting late embryonic development and hatching were investigated in a series of experiments, which were carried out in the time of the year that live biological material was available.

PART I

Scanning electron microscopy
In chapter 2 the mantle epithelium of embryos and early juveniles of the squids *L. vulgaris* and *L forbesi* and the cuttlefish *S officinalis* was studied, using scanning electron microscopy. In embryos of *L vulgaris* and *L.forbesi*, some hitherto undescribed epidermal structures were found. They are missing in *S.officinalis* embryos These so-called "extruding structures" are located at the intersection of the three branches of Hoyle's organ and first appear in developmental stage XIII of Naef. At the same stage, Hoyle's organ starts to differentiate and "uniform-type" ciliated cells become visible in the epidermis of both *L.vulgaris* and *L.forbesi* Directly after hatching the epidermis of the species examined starts to slough off and finally the extruding structures, Hoyle's organ and the ciliated cells of the mantle epithelium disappear.

Light microscopy and transmission electron microscopy
In chapter 3 the hemispheric-apex cells (HC) which form the "extruding structures" are described at the end of embryonic development, and at different times after hatching, using light microscopy and transmission electron microscopy. The morphology and presence or absence of these cells were compared with the enzyme producing cells of Hoyle's organ (CHO).

The surface membrane of the HC exhibits many microvilli. Desmosomes between HC were distributed in patches along the plasma membrane. Desmosomes were also found between HC and CHO and between neighbouring CHO. The area below the most apical desmosomes showed many cellular interdigitations. Several small Golgi zones and numerous mitochondria were found in the cell centre. Mitochondria were also abundant in the uppermost part of the cell.

In the HC the nucleus is situated near the cell centre The shape of the central area of the cell usually corresponds grossly to the nuclear form Endoplasmic reticulum was found near the nucleus and in the lowermost part of the cell, but it was never as abundant as in the CHO. In the most apical part of the HC many oval vesicles were found containing electron lucent material Ultrathin sagittal sections showed that the tapering proximal part of the HC, directed toward the underlying tissue, could be T-shaped.

As the HC, UTC and CHO degenerate after hatching, it is concluded that these structures consist of transient embryonic cells. The location of the HC on the mantle surface, in the vicinity of Hoyle's organ, the morphological changes occurring immediately after hatching and the fact that the HC undergo posthatching degeneration all suggest that they might be involved in the hatching process.

Iontophoretic injection of fluorescent dyes
In chapter 4, the morphology and intercellular communication between ectodermal cells in the mantle epithelium of *L vulgaris* and *L forbesi* were studied using iontophoretic injection of the fluorescent dyes LY (Lucifer Yellow) and LY-D (Lucifer Yellow Dextran) Four different types of ectodermal cells were injected, *viz* CHO, HC, uniform-type ciliated cells (UTC) and mucous cells (MC) No dye-coupling was found between any of these cells when LY-D was injected. When LY was used MC and UTC showed no signs of coupling, indicating their individual nature, whereas neighbouring HC and/or CHO cells appeared to be communicating. The results indicate that HC are probably activated during or just before hatching and are possibly involved in the hatching process

Cytochemical analysis of lectin labeling
Chapter 5 describes the results of a histochemical study in which fluorescein isothiocyanate (FITC) labeled lectins served as reagents to detect and characterize carbohydrate containing macromolecules in mantle epithelial cells of *S officinalis*, *L vulgaris* and *L forbesi* embryos and juveniles. It appears that the application of FITC labeled lectins is an effective method for characterizing cells and organs in decapod cephalopod embryos.

In the mantle epithelium of all species examined, CHO could very readily be distinghuished after using *Ulex europaeus* (UE I) lectin Because the UE I binding sites are probably exclusively restricted to CHO granules our investigations provide circumstantial evidence that the hatching enzyme of *L.vulgaris*, *L.forbesi* and *S.officinalis* is a α-L-fucose containing glycoprotein.

The most suitable lectin to label the HC appeared to be *Canavalia ensiformis* lectin (Con A) because it did not stain any other cell type in the mantle epithelium. The Con A binding sites of the HC were found in the cell coat and in the supranuclear area, probably inside the granules. Most likely the positive reaction can be ascribed to glucose or mannose residues. It is concluded that HC and CHO cannot only be distinguished with morphological criteria but also by their specific carbohydrate characteristics.

Biochemical studies
As described in chapter 5, hatching medium of *L.vulgaris* contains a protease which is absent in perivitelline fluid (PVF) during embryonic development and in sea water. As the enzyme is released by the embryos right before and during hatching, it can be defined as the hatching enzyme The protease has a pH optimum of 8.5 and is probably a metalloprotease The proteolytic activity was completely lost after three times freezing and thawing as well as after storage at -20°C for more than four months. Purification of *L.vulgaris* hatching enzyme from hatching medium was hampered because of the presence of a haemagglutinin in the PVF and the scarcity of crude hatching medium.

PART II

Influence of copper
The influence of copper on embryonic development and hatching of *S officinalis* is described in chapter 7. Copper exerts a profound effect on both hatching stage and time-to-hatching. At high copper concentrations (50-200 ppb), the embryos hatch at an earlier stage than the controls, but they have a lower survival potential No external morphological malformations were found. Whereas copper does not accumulate in the embryo or in the vitellus, it is absorbed by the envelope and/or chorion.

Influence of light
The influence of photoperiodicity on hatching of *L.forbesi* and *L.vulgaris* embryos was investigated under different experimental light-dark (LD) conditions. The results of this study are described in chapter 8.

The transition from light to dark stimulated hatching and functions as a "Zeitgeber" or synchronizer. Independent of the timing and duration of the dark period most embryos hatched soon after termination of the light period. Embryos which had developed in continuous light, showed no hatching rhythm at all. When exposed to a dark shock most embryos hatched soon after the onset of darkness. A twilight shock, in which the light was reduced by 50% (*i e.* 50 $\mu E\ s^{-1}\ m^{-2}$) failed to stimulate hatching. Embryos which were kept from stage X on in an artificially controlled LD cycle, preferentially hatch in a period which coincides with the period at which darkness usually occurred when placed under continuous illumination from stage XX onwards.

In chapter 9 the influence of photoperiodicity on hatching of *S.officinalis* is described. For an interpretation of the results some properties of the PVF and the egg capsule during embryonic development were taken into consideration

Like in *L.vulgaris* and *L.forbesi*, the transition from light to dark acts as a Zeitgeber in *S.officinalis* embryos. The embryos preferred to hatch during darkness, even when the dark period was short (1-4 hrs) and replaced part of the natural light period. The hatching rhythm was also independent of the embryonic stage in which the experiment was started. LD rhythmicity was never conserved. In the absence of any external LD rhythm the time-to-hatching increased. Lack of pigmentation in the egg envelope strongly decreased the time-to-hatching When embryos were exposed to a single dark period of 1 to 4 hrs, many of them hatched during darkness. On the contrary, during a dark-shock of 10 minutes hatching never took place.

When embryonic development is near completion, the egg capsule of *S.officinalis* becomes thinner as a result of the expanding PVF. Absence of the envelope did not affect embryonic development In *Sepia* the envelope has mainly a consolidating function. It compensates the osmotic pressure of the PVF. Spectrophotometrical investigations indicated that light between 200 and 900 nm is absorbed by the envelope and by ink from a female adult. The role of the black pigment in the envelopes remains obscure

Influence of salinity

The effect of salinity on embryonic development of *S.officinalis* in the Delta Area (South Western part of The Netherlands) is described in chapter 10. The investigations were carried out during 1988/1989, and the results are compared with data concerning the distribution of *S.officinalis* in the three main parts of this area, called Oosterschelde, Westerschelde and Grevelingen.

Embryos hatched in water collected at Yerseke (Oosterschelde), Vlissingen (Western part of the Westerschelde) and Bommenede (Grevelingen), *i.e.* at salinity values above 28.1 ‰, but not in water sampled at Hoedekenskerke and Hansweert (Middle and Eastern part of the Westerschelde where the salinities are below 22.0 ‰) Under laboratory conditions, using diluted Oosterschelde water, the highest hatching percentages of *S.officinalis* were found at salinities above 29.8 ‰ Some embryos hatched at a salinity value of 26.5 ‰ but hatching never occurred at salinities below 23.9 ‰ In embryos exposed to salinity changes during late embryonic development the developmental rate decreased at 28.7 ‰ salinity or less. Below 22.4 ‰ malformations occurred.

Starting at stages X-XII (Naef) the effect of abrupt salinity changes on embryonic development and hatching of *L.vulgaris* embryos from the Oosterschelde (S ± 31 ‰) was investigated. This study is described in chapter 11.

During the experiments no difference in salinity value was found between the PVF and the surrounding sea water. When the salinity of the water was abruptly reduced to 28.7 ‰, hatching and survival percentages were still high but below 28.7 ‰ the hatching percentages dropped.

Only a few embryos developed normally after being exposed to salinities between 22 and 25 ‰. In embryos with deranged development abnormalities in mantle-length were most striking. Embryos kept at 19 ‰ salinity, after six days showed mantle deformations and many developed enormous bulbs on the head. Invariably deformed embryos showed internal malformations, except in the eye and the statocyst. The internal yolk sac was grossly enlarged. No hatching took place at 19 ‰ salinity.

On the whole our results permit to conclude that salinity is an important factor limiting the distribution of *S.officinalis* and *L.vulgaris* in most parts of the Delta Area with the exception of the Western part of the Westerschelde and the Grevelingen.

In het eerste gedeelte van dit proefschrift worden zowel de morfologische studie van embryonale, ectodermale differentiaties als het biochemische onderzoek van het hatching enzym van de cephalopoden *Loligo vulgaris*, *Loligo forbesi* en *Sepia officinalis* beschreven. In het tweede deel wordt nader ingegaan op de effecten die drie oecologische factoren, met name kopergehalte van het water, licht en saliniteit, op de late embryonale ontwikkeling en hatching kunnen hebben. De in dit laatste gedeelte beschreven experimenten zijn uitgevoerd tijdens de perioden waarin levend biologisch materiaal beschikbaar was.

DEEL I

Scanning electronenmicroscopie
Hoofdstuk 2 begint met een scanning electronenmicroscopische studie van het mantelepitheel van *L vulgaris*, *L.forbesi* en *S.officinalis* embryo's en juvenielen In embryo's van zowel *L.vulgaris* als *L forbesi* werden tot nu toe onbekende epidermale structuren ondekt. Deze structuren komen niet voor in embryo's van *S.officinalis*. De zogenaamde "extruding structures" bevinden zich rond het centrum van het orgaan van Hoyle en worden voor het eerst zichtbaar in Naef's stadium XIII In hetzelfde embryonale stadium kan men, in de epidermis van zowel *L.vulgaris* als *L.forbesi*, ook het orgaan van Hoyle en de "uniform-type" geciliëerde cellen onderscheiden Direct na hatching begint de epidermis van de onderzochte soorten te degenereren en verdwijnen de "extruding structures", het orgaan van Hoyle en de geciliëerde cellen van het mantelepitheel.

Lichtmicroscopie en transmissie electronen microscopie
De hemisferische cellen (HC), welke de "extruding structures" vormen, worden met behulp van lichtmicroscopie en transmissie electronenmicroscopie in hoofdstuk 3 beschreven. Zowel de morfologie als de aan- of afwezigheid van de HC en van de enzym producerende cellen van het orgaan van Hoyle (CHO) werden aan het einde van de embryonale ontwikkeling en op verschillende tijdstippen na het uitkomen van het embryo, vergeleken.

Het hemisferisch boven de epidermis uitstekende gedeelte van de HC vertoont vele microvilli. Tussen de HC bevinden zich op verschillende plaatsen desmosomen. Deze desmosomen werden ook aangetroffen tussen HC en CHO en tussen CHO onderling In het gebied direct onder de meest apicaal gelegen desmosomen, vertoont de celmembraan vele vingervormige vertakkingen In het centrum van de HC werden gedeelten van het Golgi apparaat en talrijke mitochondrien aangetroffen. Mitochondria werden ook gevonden in het meest apicale gedeelte van de cel.

De kern van de HC is gelokaliseerd in het centrum van de cel. Deze is meestal peervormig en correspondeert met de vorm van het celcentrum. Endoplasmatisch reticulum werd, echter in minder grote hoeveelheid dan in de CHO, aangetroffen rondom de kern en in het meest basale gedeelte van de HC. In het hemisferische gedeelte van de HC werden talrijke vesikels met electronen transparante inhoud aangetroffen. In ultradunne sagittale coupes heeft het smal uitlopende proximale gedeelte van de HC een T-vorm.

De HC, CHO en UTC zijn transitoire embryonale cellen die na hatching degenereren. De ligging van de HC rond het centrum van het orgaan van Hoyle, de morfologische veranderingen die de cellen vertonen direct na hatching alsmede het feit dat de HC degenereren, suggereren dat de HC betrokken zijn bij het uitkomen van het embryo.

Iontoforetische injecties van fluorescerende merkstoffen
In hoofdstuk 4 wordt de morfologie en de celcommunicatie tussen ectodermale cellen in het embryonale mantelepiteel van *L vulgaris* en *L.forbesi* onderzocht, gebruik makend van iontoforetische injectie van de fluorescente merkstoffen LY (Lucifer Yellow) en LY-D (Lucifer Yellow Dextran). Er werden vier verschillende celtypen geïnjecteerd met name CHO, HC, "uniform-type" geciliëerde cellen (UTC) en slijmcellen (MC). Dye-coupling werd niet waargeno-

men wanneer de cellen werden ingespoten met LY-D. Indien gebruik werd gemaakt van LY, vertoonden de MC en de UTC geen dye-coupling Wel leken de HC en CHO verbonden te zijn. De resultaten geven aan dat de HC mogelijk vlak vóór en/of gedurende het hatchingproces geactiveerd worden en dus betrokken zouden kunnen zijn bij het uitkomen van het embryo.

Histochemisch onderzoek met behulp van lectinen
Hoofdstuk 5 beschrijft de resultaten van een histochemische studie waarbij gebruik werd gemaakt van met FITC gemerkte lectinen. De experimenten toonden aan dat met behulp van deze markers de verschillende cellen en organen in decapode cephalopoden embryo's goed kunnen worden onderscheiden.

In alle onderzochte soorten konden de CHO zonder moeite worden onderscheiden in het mantelepitheel, indien gebruik werd gemaakt van het lectine van *Ulex europaeus* (UE I). Het hatching enzym van *L.vulgaris*, *L.forbesi* en *S.officinalis* bezit mogelijk α-L-fucose bevattende glycoproteinen omdat UE I bindingsplaatsen alléén aangetroffen werden in de granula van de CHO

Het lectine van *Canavalia ensiformis* (Con A) bleek het meest geschikt voor het merken van de HC. De Con A bindingsplaatsen werden aangetroffen in de extracellulaire laag tussen de microvilli ("cell coat") en in het apicale celgedeelte boven de kern. Waarschijnlijk kan de positieve reactie worden toegeschreven aan glucose en mannose residuen. Uit de resultaten kan worden geconcludeerd dat de HC en de CHO niet alleen kunnen worden onderscheiden op basis van morfologische criteria maar ook op basis van cel-specifieke carbohydraten.

Biochemische studies
Zoals wordt beschreven in hoofdstuk 5 bevat hatching medium van *L.vulgaris* een protease dat niet voorkomt in perivitelliene vloeistof (PVF) gedurende de embryonale ontwikkeling en ook niet in zeewater. Omdat het enzym vlak vóór en/of tijdens hatching afgegeven wordt, kan worden aangenomen dat dit protease het hatching enzym is. Het enzym vertoont optimale activiteit bij pH 8.5 en is waarschijnlijk een metalloprotease. De proteolytische activiteit gaat echter verloren na drie maal bevriezen en ontdooien of wanneer het enzym langer dan vier maanden bij -20°C wordt bewaard. De zuivering van het enzym werd voornamelijk bemoeilijkt door de aanwezigheid van een haemagglutinine in het PVF en door de beperkte beschikbaarheid van hatching medium.

DEEL II

Invloed van koper
De invloed van koper op de embryonale ontwikkeling en hatching van *S.officinalis* is beschreven in hoofdstuk 7. Koper beinvloedt zowel het embryonaal hatching stadium als het tijdstip waarop hatching plaatsvindt. Ten opzichte van de controlegroepen komen embryo's vroeger uit in de embryonale ontwikkeling en hebben ze een kleinere overlevingskans wanneer ze worden blootgesteld aan hoge koper concentraties (50-200 ppb). Deze embryo's vertoonden echter geen uitwendig zichtbare morfologische afwijkingen Koper wordt voornamelijk geabsorbeerd door het eikapsel en derhalve niet opgeslagen in het embryo of in het dooiermateriaal.

Invloed van licht
De invloed van fotoperiodiciteit op hatching van *L.forbesi* en *L.vulgaris* is bestudeerd onder verschillende experimentele licht-donker (LD) condities. De resultaten van deze studie zijn weergegeven in hoofdstuk 8.

De overgang van licht naar donker stimuleerde hatching en functioneerde als een "Zeitgeber". De meeste embryo's kwamen vrij snel uit nadat de lichtperiode beeindigd was, onafhankelijk van de duur en het tijdstip van de donkerperiode. Embryo's die waren blootgesteld aan ononderbroken licht vertoonden geen specifiek hatchingritme. Embryo's welke een donkerschok hadden ervaren verlieten hun omhulsels direct na de overgang van licht naar donker. Hatching werd niet gestimuleerd wanneer de embryo's werden blootgesteld aan een schemerlichtschok (reductie van de lichtintensiteit tot de helft, d.i. 50 $\mu E\ s^{-1}\ m^{-2}$) Embryo's, welke vanaf stadium X blootgesteld zijn geweest aan een artificieel gecontroleerd LD ritme, en in stadium XX continu belicht werden,

vertoonden een hatchingritme overeenkomstig de geëlimineerde donkere periode.

Hoofdstuk 9 beschrijft het effect van fotoperiodiciteit op hatching van *S.officinalis*. Bij de interpretatie van de resultaten werd rekening gehouden met enkele onderzochte eigenschappen van het PVF en het eikapsel gedurende de embryonale ontwikkeling van *Sepia*.

Zoals bij *L.vulgaris* en *L.forbesi* functioneert de overgang van licht naar donker als "Zeitgeber". Embryo's kwamen bij voorkeur gedurende de donkerperiode uit, zelfs wannneer deze periode erg kort was of was verplaatst naar de oorspronkelijke lichtperiode. Het hatchingritme werd niet beïnvloed door het embryonaal stadium bij aanvang van het experiment. De LD ritmiek werd echter nooit geconserveerd. Bij afwezigheid van elk willekeurig LD ritme werd het tijdstip van hatching vertraagd Embryo's, met weinig of niet gepigmenteerde omhulsels, kwamen eerder uit dan embryo's omgeven door zwarte eikapsels. Vrijwel alle embryo's verlieten hun omhulsels gedurende een donkerschok indien ze werden blootgesteld aan één tot vier uur duisternis. Gedurende donkerschokken van tien minuten vond nooit hatching plaats.

Door de toenemende hoeveelheid PVF gedurende de embryonale ontwikkeling is het eikapsel van *S officinalis* in het hatching stadium veel dunner dan in de vroegere stadia van de ontwikkeling Indien de zwarte buitenlaag wordt verwijderd wordt de embryonale ontwikkeling niet nadelig beïnvloed. Deze laag heeft waarschijnlijk alleen een verstevigende functie en compenseert de osmotische druk van het PVF Spectrofotometrische studies toonden aan dat licht tussen 200 en 900 nm wordt geabsorbeerd door zowel de buitenlaag van zwarte eieren als door inkt afkomstig van een volwassen *Sepia* wijfje. De functie van het zwarte pigment blijft vooralsnog onduidelijk.

Effect van saliniteit

Het effect van saliniteit op de embryonale ontwikkeling van *S.officinalis* in het Delta gebied (provincie Zeeland, Nederland) wordt beschreven in hoofdstuk 10. De experimenten vonden plaats in 1988/1989 en de resultaten werden vergeleken met gegevens omtrent de verspreiding van *Sepia* in de drie belangrijkste watergebieden Oosterschelde, Westerschelde en de Grevelingen

Embryo's kwamen uit in water met een saliniteitswaarde hoger dan 28.1 ‰ dat gemonsterd werd bij Yerseke (Oosterschelde), Vlissingen (westelijk deel van de Westerschelde) en Bommenede (Grevelingen) maar niet in water met een zoutgehalte lager dan 22.0 ‰ verkregen bij Hoedekenskerke en Hansweert (het centraal en oostelijk gedeelte van de Westerschelde).

Gebruik makend van verdund Oosterschelde water werden onder laboratoriumcondities de hoogste hatchingpercentages gevonden in water met saliniteitswaarden hoger dan 29 8 ‰. Sommige embryo's kwamen uit bij een zoutgehalte van 26.5 ‰. Hatching vond niet plaats bij saliniteitswaarden onder 23.9 ‰.

Vanaf stadium X-XII (Naef) werd het effect van abrupte saliniteitsveranderingen op hatching van *L.vulgaris*, afkomstig uit de Oosterschelde, bestudeerd. Dit onderzoek is beschreven in hoofdstuk 11.

Gedurende de experimenten werden geen verschillen gevonden tussen de saliniteitswaarden van PVF en zeewater. Hoge hatching- en overlevingspercentages werden genoteerd wanneer het zoutgehalte van het water abrupt werden verlaagd tot 28.7 ‰ Bij lagere waarden vond een sterke daling van de hatchingpercentages plaats.

Slechts enkele embryo's ontwikkelden zich normaal wanneer ze werden blootgesteld aan abrupte saliniteitsveranderingen tussen 22 en 25 ‰. In abnormaal ontwikkelde embryo's werd veelal een afwijkende mantellengte aangetroffen.

Embryo's kwamen niet uit wanneer ze abrupt werden blootgesteld aan een saliniteit van 19 ‰. Al zes dagen na het starten van het experiment vertoonden de meeste van deze embryo's opvallende manteldeformaties en grote blazen op de kop. Alle abnormaal ontwikkelde embryo's vertoonden ook afwijkingen van de interne structuren, met uitzondering van de ogen en de statocysten De interne dooierzak was sterk vergroot.

Uit de resultaten van de saliniteitsexperimenten mag geconcludeerd worden dat saliniteit waarschijnlijk één van de belangrijkste factoren is die de verspreiding van zowel *L.vulgaris* als *S officinalis* in de meeste delen van het Delta gebied, met uitzondering van het westelijk deel van de Westerschelde en de Grevelingen, reguleert.

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De schrijfster van dit proefschrift werd op 27 mei 1962 geboren te Eindhoven. In 1978 en 1980 behaalde zij achtereenvolgens de diploma's voor het Middelbaar en Hoger Algemeen Voortgezet Onderwijs (Gemeentelijke Scholengemeenschap Woensel te Eindhoven). Vanaf augustus 1980 studeerde zij aan de Hogeschool Katholieke Leergangen te Tilburg (Moller Instituut) en behaalde in februari 1985 de tweede-graads leraren bevoegdheid in de vakken biologie en voedingsleer/ huishoudkunde (afstudeerstage aan Katholieke Universiteit Brabant, afdeling histologische psychologie, begeleiders Prof Dr. J. Weynen en Drs J Wouters). Van maart tot en met september van dat jaar was zij tijdelijk werkzaam als intercedente bij Randstad Uitzendburo te Eindhoven. In september 1985 werd aangevangen met de doctoraal studie biologie aan de Katholieke Universiteit te Nijmegen. Daarnaast volgde zij een V.W.O. cursus Natuurkunde aan de Avondscholengemeenschap Craneveldt te Nijmegen welke in augustus 1987 werd afgesloten met het behalen van het colloquium doctum. Ten behoeve van haar doctoraal hoofdvak Ontwikkelingsbiologie was zij in 1986 gedurende enkele maanden werkzaam op het Station Marine te Wimereux (Frankrijk). In april 1989 behaalde zij het doctoraal examen biologie (Fysiologisch-Biochemische richting). Van april 1987 tot 1991 was zij tevens als wetenschappelijk medewerkster (A.I.O) werkzaam op de afdeling Zoologie I (vakgroep Experimentele Dierkunde) van de Faculteit Natuurwetenschappen aan de Katholieke Universiteit te Nijmegen (de doctoraal studie werd derhalve van september 1987 tot 1988 onderbroken). Als A.I.O heeft zij een bijdrage geleverd aan het H.B.O. en doctoraal biologie onderwijs (practicum en voorcolleges ontwikkelingsbiologie voor 2e jaars doctoraal studenten, verzorgen research practica voor 3e jaars doctoraal studenten, begeleiden en beoordelen stage hoofdvak ontwikkelingsbiologie, gastcolleges Katholieke Leergangen te Tilburg en stage begeleiding van studenten van deze opleiding). Daarnaast verzorgde ze colloquia voor vakgenoten op het Delta Instituut voor Hydrobiologisch Onderzoek te Yerseke. Tevens volgde zij aanvullend postdoctoraal onderwijs (NATO-ASI cursus 1989, "Experimental Embryology in Aquatic Plant and Animal Organisms" te Banyuls-sur-Mer in Frankrijk (coordinator Dr. H.J. Marthy); studie "Neuroanatomie" te Oxford (Prof. J.Z. Young); literatuurstudie "Endocriene Organen in Cephalopoden (Prof. Dr. J.M Denucé); cursus "Cell Communication" (Prof. Dr. S.E. Wendelaar-Bonga, Dr. B.J. Jenks en Dr. F. van Herp), cursus Managment voor A.I.O.'s (Prof. K. Verhoeff/ Drs. J.G.J. van de Broek); cursus Schrijven over Wetenschap (Dr J T.J.M. Willems); Lustrumcolleges K.U. te Nijmegen "Leven in Genen Delen"). Gedurende dezelfde periode is het wetenschappelijk onderzoek verricht dat in dit proefschrift beschreven is. Voor het experimentele gedeelte van dit onderzoek bracht zij werkbezoeken aan het Plymouth Marine Laboratory (Groot Brittannie) en het Stazione Zoologica "Antonio Dohrn" te Napels (Italie). Daarnaast was zij gedurende enkele maanden per jaar werkzaam op het Delta Instituut voor Hydrobiologisch Onderzoek te Yerseke.

1) De vergelijking van minimale saliniteitstoleranties voor embryo's van *Sepia officinalis* met *Sepia subaculeata* door Palmegiano en D'Apote (1983) is onjuist.

Referenties: Choe, S. (1966) On the eggs, rearing, habits of the fry, and growth of some cephalopoda. Bull. Mar Sci. 16: 330-348.

Palmegiano, G.B. and M P D'Apote (1983). Combined effects of temperature and salinity on cuttlefish (*Sepia officinalis* L.) hatching. Aquaculture 35: 259-264.

2) Bij het beschrijven van posthatching stadia van cephalopoden is het beter van juvenielen te spreken dan van larven.

Referentie. Von Boletzky, S. (1974) The "larvae" of Cephalopoda. A review. Thalassia Jugosl. 10: 45-76.

3) Indien aangenomen wordt dat voor een funktie slechts weinig sollicitanten geschikt zullen zijn, is het standaardgebruik van intelligentie- en persoonlijkheidstesten voor de selectie van deze kandidaat af te raden.

referentie: Afstudeerrede door L Kuijk (Katholieke Hogeschool Tilburg).

4) De buiten-contractuele schadeplichtigheid voor de veroorzaker van schade aan het milieu dient naar huidig recht niet gebaseerd te zijn op schuld- maar op risico-aansprakelijkheid.

referentie: Doctoraalscriptie Mr. A.J Kromhout "Een beschouwing van een "gevaarloze" regeling voor gevaarlijke stoffen", Katholieke Universiteit Nijmegen, Faculteit der Rechtsgeleerdheid.

5) Het Nederlands overheidsbeleid 1990/1991 met betrekking tot het selecteren van geschikte kandidaten voor een vacature zal voor de vrouwenemancipatie nadelige gevolgen hebben.

6) De mening van vele doctoraal studenten biologie, dat techniek moet worden verheven boven onderzoek, is een onjuiste visie op wetenschap.

7) There is no *best* way to investigate biological problems.